The applications of RNA analysis on identification of body fluids and dating plucked hairs

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Abstract

The aim of this study is to explore several possible uses of RNAs in forensic science, including body fluid identification using LAMP (loop-mediated isothermal amplification) technology and dating the plucked hair by real-time PCR. A novel application of real-time reverse transcription LAMP to identify the presence of a specific body fluid using blood and saliva is reported as a proof-of-concept model in this study. In comparing with other recently developed methods of body fluid identification, the RT-LAMP assay is more rapid and requires only one simple heating-block maintained at a single temperature, circumventing the need of dedicated equipment. The *18S* rRNA locus was used as the internal control, and haemoglobin beta (*HBB*) and histatin 3 (*HTN3*) as the blood-specific and saliva-specific marker respectively. The data showed that the specificity and the limit of detection was a minimum of 10^{-5} ng total RNA for detection of both *18S* rRNA and *HBB*, whereas it was 1.25ng total RNA for detection of *HTN3*. The detection of RT-LAMP products was performed by separation of the products using gel electrophoresis and collecting the fluorescence of calcein. The data collected indicated complete concordance with the body fluid tested regardless of the detection method used. This is the first application of real-time RT-LAMP to detect body fluid-specific RNA and demonstrates the use of this method in forensic biology.

The determination of the time since a hair was plucked was estimated by the analysis of *18S rRNA* and *ß-actin* mRNA using real-time PCR. The difference of average Cq value (dCq between the Cq values of *18S rRNA* and *ß-actin* mRNA was monitored over a period of 8 weeks. The data presented that the age of the plucked hair could be approximately estimated using a liner polynomial: the time since the hair was plucked (TSP)= 0.168 × dCq + 13.406 (r=0.99) with a limitation of 21 days. It was also

noticed that some factors, such as the uncertainty of the actual phase of the plucked hairs, chemicals used for hair dye or perm, and other uncontrolled environmental conditions, may impact the estimation.

Acknowledgements

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謹以這篇論文獻給我的父親！

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List of Abbreviations

18S rRNA ····· 18S ribosomal RNA

ACP ····· Acid phosphatase

BCP ····· Bromochloropropane

ACTB ····· ß-actin

BIP ····· Backward inner primer

cDNA····· Complementary deoxyribonucleic acid

Cq ····· Quantification cycle

Ct ····· Threshold cycle

DEPC ····· Diethylpyrocarbonate

DNA ····· Deoxyribonucleic acid

DNase ····· Deoxyribonuclease

dNTP ····· Deoxyribonucleotide triphosphate

EtBr····· Ethidium bromide

FIP····· Forward inner primer

GAPDH····· Glyceraldehyde-3-phosphate dehydrogenase

HBB····· Haemoglobin subunit beta

HTN3····· Histatin 3

HV I ····· Hypervariable region I

HVII ····· Hypervariable region II

KM test ····· Kastle-Meyer test

KRT13····· Keratin 13

KRT4····· Keratin 4

KRT6A ····· Keratin 6A

LAMP ····· Loop-mediated isothermal amplification

LR ····· Likelihood Ratio

LR+····· Positive Likelihood Ratio

LR-····· Negative Likelihood Ratio

miRNAs····· Micro ribonucleic acids

mRNA ····· Messenger ribonucleic acid

mtDNA····· Mitochondrial DNA

NMD ····· Nonsense-mediated mRNA decay

NPV····· Negative Predictive Value

PBGD ····· Porphobilinogen deaminase

PBS····· Phosphate buffered saline

PCR ····· Polymerase chain reaction

PPV ····· Positive Predictive Value

PRM1 ····· Protamine 1

PRM2 ····· Protamine 2

PSA····· Prostate-specific antigen

RMP ····· Random match probability

Real-time RT-LAMP····· Real-time reverse transcription loop-mediated isothermal amplification

RFLP ····· Restriction fragment length polymorphism

RNA····· Ribonucleic acid

RNase ····· Ribonuclease

rRNA ····· Ribosomal ribonucleic acid

RT-LAMP ····· Reverse transcription loop-mediated isothermal amplification

RT-PCR ····· Reverse transcription polymerase chain reaction

SPRR1A ····· Small proline rich protein 1A

SPRR2A ····· Small proline rich protein 2A

SPTB····· Spectrin beta

SSRs ····· Simple sequence repeats

STATH····· Statherin

STRs ····· Short tandem repeats

Tm····· Melting temperature

TMB ····· Tetramethylbenzidine

TMB test ····· Tetramethylbenzidine test

tRNA ····· Transfer ribonucleic acid

TSP····· Time since the hair was plucked

Tt ····· Threshold time

VNTR····· Variable number of tandem repeats

Chapter 1 Introduction

According to “Locard’s exchange principle” [1], trace evidence is transferred between suspects and victims and therefore can be found at crime scenes. It is important to identify the source of the evidence, which includes biological evidence (such as a blood stain), physical evidence (such as a fingerprint), chemical evidence (such as a drug), temporary or circumstantial evidence (such as a broken door) [2]. Biological evidence found at the crime scene includes body fluids (such as blood, semen, and saliva), hair, skin, bone, or tissue. Among them, body fluids are the most common evidence found at the crime scene, especially in violent crimes. Thus, it is very important for criminal investigation to identify the donor as well as the type of these body fluids.

Blood, semen and saliva are common body fluids collected at the crime scene. Sometimes vaginal secretions, menstrual blood, urine, and sweat may be found in some particular crime cases, such as sexual assault cases [3]. Traditionally, many types of body fluid identification methods, such as chemical tests, immunological tests, protein catalytic activity tests, spectroscopic methods and microscopy are used in forensic community to deal with body fluids [4]. These methods are relatively simple and straightforward. However, conventional serology-based methods for body fluid identification are prone to various limitations, such as sample amount [5]. Later, with the technological advancement in molecular biology, DNA research has predominated in forensic biology in the past three decades [6], especially DNA profiling. Body fluids contain valuable DNA information to identify the donator of the body fluids, which can help investigators identify suspects, victims or innocent individuals. However, DNA profiling can help only for comparison between the evidence and its donator but not for determination of the type and origin of the body fluids, which is also crucial in

criminal investigation. Recently, RNAs has been introduced to forensic community for this purpose due to its specificity for different body fluids [7]. The analysis of cell-specific mRNA expression has been proposed as a promising new method for body fluid identification. In the following, common human body fluids and the methods traditionally used to identify these body fluids by forensic scientists were introduced, followed by DNA analysis and then RNA analysis developed later.

1.1 Human body fluids and the traditional identification methods

Confirming body fluid type is an important and the first step of DNA identification process in the laboratory. Some body fluids have special colour or odour that may be used to determine their type (see the following sections). However, it is such a subjective evaluation with questionable accuracy that may mislead the investigation. Thus, it is necessary to use a scientifically confirmatory test for the body fluids. Kelly Virkler *et al*. have listed out the most common body fluids and their components, including blood, semen, saliva, vaginal secretion, urine and sweat [3] as shown in Table 1-1.

The identification of biological fluids is mainly accomplished with chemical, serological, or immunological analysis for presumptive and confirmatory testing. Although these methods are fast, there is a problem of cross reaction and therefore most of them are not specific to human samples. In addition, colour tests rely on subjective judgments to decide whether the result is positive or negative. It could be more confusing when the fluid is a mixture which is often found in the vaginal swab from the victim of the sexual assault case. There is also the problem of poor quality

samples and samples on substrates that may inhibit the reaction, thereby leading to false negative results.

Table 1-1 Composition of six common body fluids

Blood	Semen	Saliva	Vaginal secretion	Urine	Sweat
• Haemoglobin	• Acid phosphatase	• Amylase	• Acid phosphatase	• Urea	• Urea
• Fibrinogen	• Prostate-specific antigen	• Lysozyme	• Lactic acid	• Creatinine	• Lactic acid
• Erythrocytes	• Spermatozoa	• Mucin	• Citric acid	• Uric acid	• Chloride
• Albumin	• Choline	• Buccal epithelial cells	• Urea	• Chlorine	• Sodium
• Glucose	• Spermine	• Thiocyanate	• Vaginal peptidase	• Tamm-Horsfall glycoportein	• Potassium
• Immunoglobulins	• Semenogelin	• Potassium	• Glycogenated epithelial cells		• Immunoglobulins
	• Zinc	• Bicarbonate	• Acetic acid		
	• Citric acid	• Phosphorus	• Pyridine		
	• Lactic acid	• Glucose	• Squalene		
	• Fructose	• Immunoglobulins	• Immunoglobulins		
	• Urea				
	• Ascorbic acid				
	• Immunoglobulins				

Adapted from Kelly Virkler *et al.*, "Analysis of body fluids for forensic purposes: from laboratory testing to non-destructive rapid confirmatory identification at a crime scene." Forensic Science International 188.1 (2009): 1-17.

Each of the body fluids may have more than one screen test and/or one confirmatory test method. In spite of several shortcomings, some of the methods are still used by forensic scientists because they are rapid, simple, convenient, and economic [2]. The following addresses the body fluids that may be found at the crime scene as well as their presumptive and confirmatory tests.

1.1.1 Blood

Blood is the most common body fluid and quite often found at crime scenes, especially in violent crimes [8]. It can be found on the weapons, objects, walls, floor,

suspects, victims, etc. at crime scenes. It is also the most critical evidence that often leads to a breakthrough in a criminal investigation. Blood accounts for approximately 7% of an adult body weight. The average density is approximately 1060 kg/m^3 and average blood volume is roughly 5 litres for an adult. The fluid portion of blood (known as plasma which is around 55% by volume) is a kind of extracellular fluid. This extracellular fluid contains various suspended blood cells, including red blood cells (also called erythrocytes, about 45 %), white blood cells (also called leukocytes, about 1 %), and platelets (less than 1%). The average normal red blood cell count is 5.4 million/µl in men and 4.8 million/µl in women. The approximate normal range of total white blood cell is 4000 to 11000 in 1µl of blood [9].

There are 5 types of white blood cells, including neutrophils, eosinophils, basophils, lymphocytes, and monocytes. All of them have a nucleus, so both DNA and RNA can be obtained from these white blood cells. On the other hand, red blood cells are produced through erythropoiesis, developing from committed stem cells to mature erythrocytes. In mammals, the nucleus of erythrocytes has been extruded during erythropoiesis, so the mature red blood cells do not contain DNA. However, the immature red blood cells, namely reticulocytes, usually have been released into the peripheral blood from the bone marrow after extrusion of their nucleus. Thus, reticulocytes do not have a nucleus but still contain residual polyribosomes used in the formation of haemoglobin in the developing erythrocyte [10]. Besides, RNA synthesized in the previous immature stage can still be detected in human red blood cells based on gene expression analysis [11]. Meanwhile, because human blood platelets are also anucleate cells, they contain no DNA but still contain minute amounts of translational active mRNA.

Fresh bloodstains are glossy reddish-brown in colour, but the colour may change due to several factors, such as aging, environmental conditions, or other negative factors [12]. Even the surface where the blood stain is found may also play a role [13]. The colour may turn into brown, grey, or even black under the influence mentioned above, which may confuse the crime scene investigators. Besides, these factors may also cause false negative results in presumptive tests addressed below [14].

The traditional methods of blood identification include physical analysis (for example, microscopic observation), chemical analysis (for example, colour test, crystal test and spectral analysis), species analysis, and individualization analysis [2]. In physical analysis, the lack of cell nucleus in the red blood cell is a unique characteristic of vertebrates, which can be observed under microscopes. An observation of Barr body in white cells indicates the gender of the donor. Microscopic analysis of blood can also be used as a pathological analysis. For forensic blood identification, the standard procedures include presumptive test, confirmatory test, species analysis, and individualization analysis. Figure 1-1 shows the flowchart of the procedures.

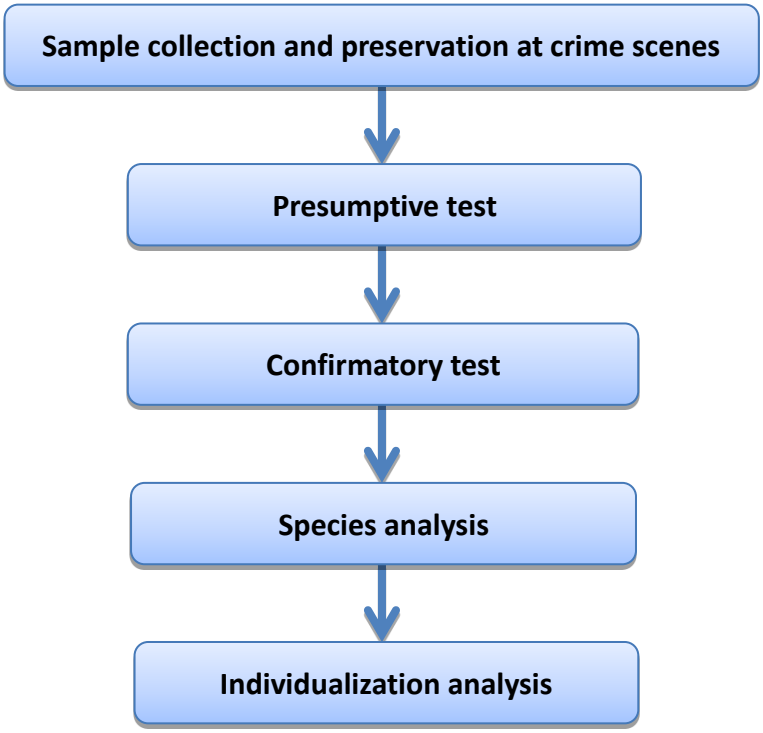


Figure 1-1 Flow chart of blood identification

When a suspected bloodstain is found at a crime scene, a presumptive test is conducted followed by a blood confirmatory test. Once the stain was identified as blood, a species analysis is then conducted to confirm whether or not it is originated from human. The last step is to perform an individualization analysis, i.e, DNA profiling.

The basic principle of most of presumptive tests for blood is based on an oxidation-reduction reaction involving the oxidant (H_2O_2) and the reductant (reagents) with the presence of the haemoglobin for its peroxidase activity. The haem group acts as a catalyst, not a reactant [15]. The basic chemical equation is shown in Figure 1-2.

$$H_2O_2 \xrightarrow{\textit{Haem-group}} H_2O + O_2$$

Figure 1-2 The principle of blood identification methods

In the reaction, the oxygen released by the haem acting as a catalyst is used to either change colour or produce colour in specific dyes that are applied to the reaction in their reduced state (Figure 1-3 to Figure 1-10). The reaction should be fast and the colour is strong enough for analysts to observe the change of colour. Thus, if colour change occurs in a short time after the reagent is added in the sample, it indicates that the sample may contain haemoglobin. The sensitivity of colour test for blood is high; however, false positive may occur since some substances could lead to same reaction as well. The source of false positive can come from chemical oxidant or catalyst, for example, cupric or nickel salt. Although positive result cannot confirm whether the sample is blood or not due to the possibility of false positive, negative result can disconfirm it. There are several common colour tests for blood, including Benzidine test, Phenolphthalein test, Leucomalachite green test, Luminol test, O-tolidine test, and Tetramethylbenzidine test [16].

(a) Benzidine test

Benzidine test is a very sensitive screening test for blood. It makes use of the peroxidise-like action of haem group of haemoglobin. The principle of this test relies on the peroxidise action of haematin. In presence of an oxidant, haem group oxidizes colourless Benzidine to a blue compound (Figure 1-3). Benzidine is a highly carcinogenic reagent, so it is not recommended to be used as an indicator of colour test for blood now [17, 18].

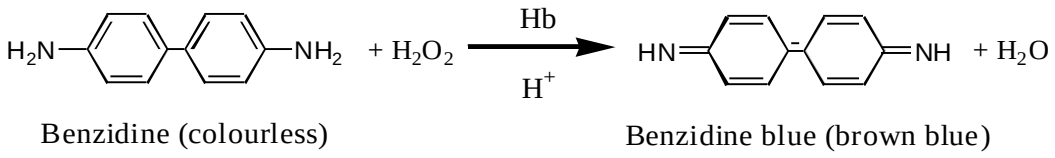


Figure 1-3 The structural formula and reaction of Benzidine test

(b) Phenolphthalein test

Phenolphthalein test, also referred to as Kastle-Meyer test (KM test), is frequently used as a forensic presumptive blood test. It was first presented in 1903, in which the chemical indicator phenolphthalein is used to detect the possible presence of haemoglobin (Figure 1-4 and Figure 1-5). The reagent is ideal to be kept in zinc powder to prevent from oxidization. Phenolphthalein reagent is extremely sensitive (produced 100 % positive results down to 10^{-3} dilution and 54.4 % positive results at 10^{-7} dilution [19]) among the presumptive tests for blood. Nevertheless, it is time-consuming to prepare the reagent and the reagent cannot be stored for an extended time [20, 21].

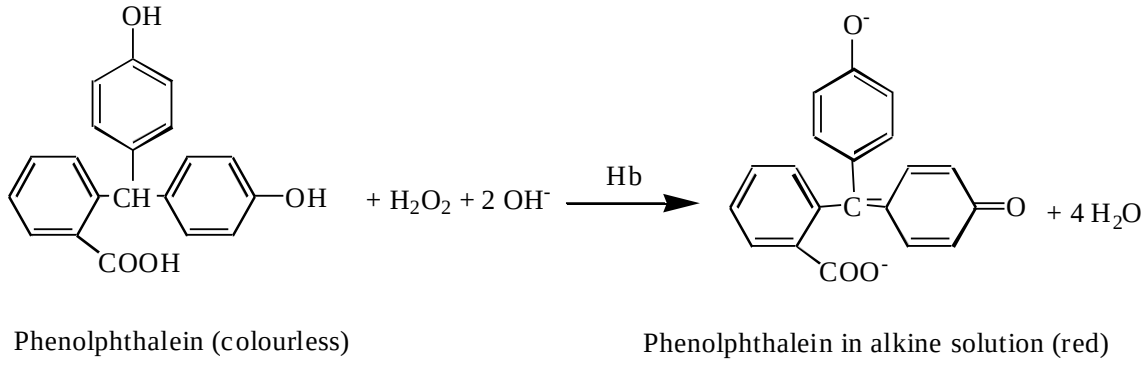


Figure 1-4 The structural formula and reaction of Phenolphthalein test

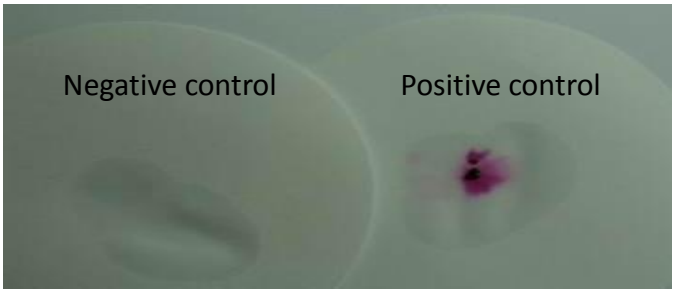


Figure 1-5 The Phenolphthalein test in positive and negative control

The sample on the right indicates a positive result of a bloodstain (around 20µl). The sample on the left indicates a negative result.

(c) Leucomalachite green test

This presumptive test is based on the peroxidase-like activity of haemoglobin of blood [22]. Leucomalachite green (4,4'-Tetramethyldiaminotriphenylmethane) is oxidized by hydrogen peroxide in the presence of peroxidases. Leucomalachite green is one of the most specific presumptive methods for blood [21]. Blood can still be detected by this method after 1/10,000X dilution [3, 23]. Reducing agents could be capable of interfering in the presumptive test. If a blue-green colour appears right after applying 1-2 drops of leucomalachite green reagent to the sample, it indicates the existence of a chemical oxidant in the sample. Hence, the test should be considered to be inconclusive. If there is no colour change in this step, then add 1-2 drops of 3% hydrogen peroxide to the sample. The appearance of a blue-green colour represents a positive result (Figure 1-6).

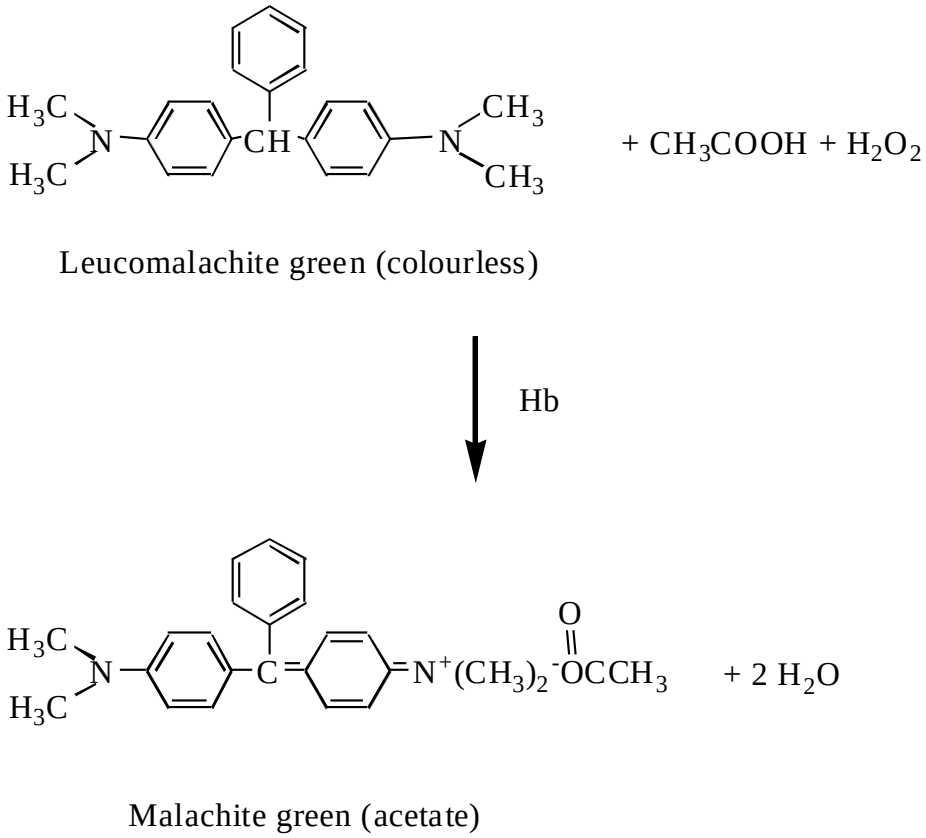


Figure 1-6 The structural formula and reaction of Leucomalachite green test

(d) Luminol test

Luminol (3-aminophthalhydrazide) is used by forensic scientists to search for latent blood trace that cannot be observed by the naked eye. When luminol comes in contact with haemoglobin in the blood, a chemiluminescence would appear (Figure 1-7). Bloodstains are still detectable by using luminol even if they have been wiped and/or cleaned. Luminol test can be used to identify the location of bloodstains at a large-scale crime scene to facilitate investigation. It can also be used to evaluate blood pattern [20, 24, 25]. Garofano *et al.* represented that blood can still be detected by luminol after 1/100,000 to 1/10,000,000X dilution [26]. Moreover, Ann Marie Gross *et al.* represented that luminol test does not have adverse effects on PCR testing, so the luminol-containing samples can still be used for further DNA profiling [27]. However,

it was also shown that luminol-treated bloodstains resulted in stutter peaks and suggested that luminol may influence PCR amplification [28]. The drawbacks of luminol test are that false positive can occur in the presence of copper ions or bleach, and the spraying of luminol reagent may introduce contamination among evidences at crime scenes. This test is also restricted as it can only be conducted in a dark environment, such as an indoor crime scene. Besides, another drawback is that spraying luminol often washes off the DNA and disrupts the original blood stain patterns.

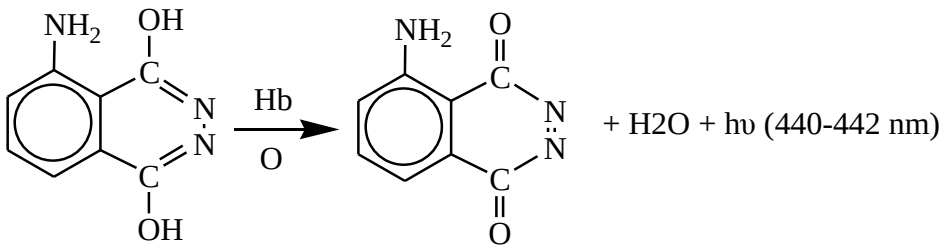
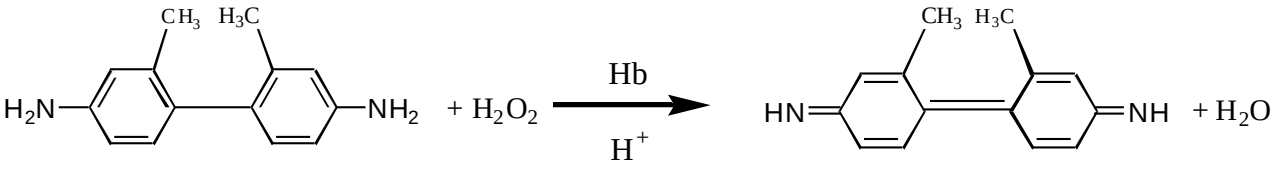


Figure 1-7 The structural formula and reaction of Luminol test

(e) O-tolidine test

O-tolidine was commonly used in colour tests for blood (Figure 1-8 and Figure 1-9) [21]. It is inexpensive and easily prepared. However, as it is carcinogenic, this reagent is no longer used in most forensic laboratories.



(Colourless) (Green)

Figure 1-8 The structural formula and reaction of O-tolidine test

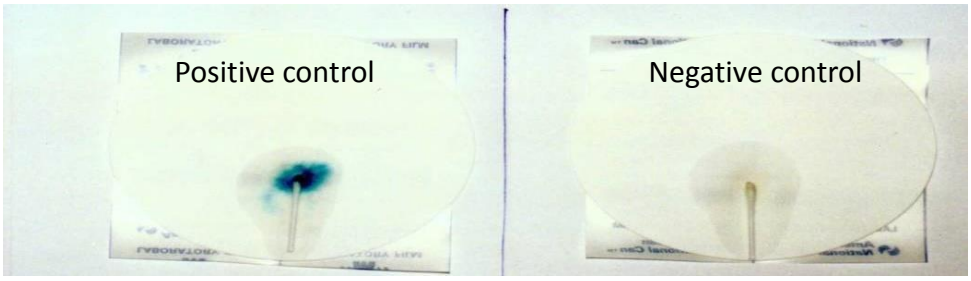
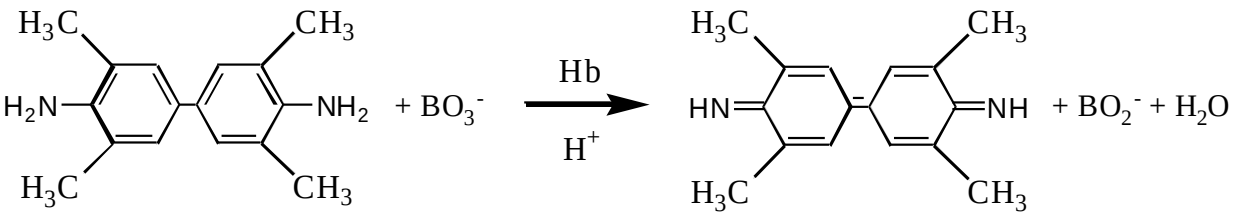


Figure 1-9 The O-tolidine test in positive and negative control

The sample on the left indicates a positive result of blood on a cotton swab (around 20µl) while the sample on the right indicates a negative result.

(f) Tetramethylbenzidine (TMB) test

Holland *et al*. reported the synthesis of TMB and suggested its potential for the detection of blood (Figure 1-10) [29]. TMB is less carcinogenic than benzidine and O-tolidine [21, 30]. It is a highly sensitive presumptive test used to determine whether a suspected stain is blood or not.



Tetramethylbenzidine (colourless) Oxidized tetramethylbenzidine (green)

Figure 1-10 The structural formula and reaction of Tetramethylbenzidine test

After a positive presumptive colour test, the confirmative test is then applied. The primary confirmative methods include crystal test, spectral analysis, electrophoresis analysis, and chromatographic analysis. Teichmann [31] and Takayama [32] tests are the most commonly used crystal test methods. A positive result confirms the sample as blood, but a negative result may occur if only a trace of blood is present or if the bloodstain is too old, making it difficult for the crystals to form [31, 32].

Once a sample is presumed to be blood, it must also be confirmed and determined if it is human blood or not. Forensic scientists usually try to utilise double immunodiffusion method with human specific antibody. Conjugated complexes between tested sample and the human specific antibody are formed if the blood comes from human; therefore, a result of conjugated complexes would confirm human blood. Immunochromatographic test (Figure 1-11) for human blood identification with commercial kits is also available (Figure 1-12) [33-35]. Blood can still be detected by this method after 1/100,000X dilution [33]. For example, Rapid Stain Identification of Human Blood (RSID™-Blood) is a commercial kit targeting glycophorin (GYPA) which is one of the major sialoglycoproteins of the human erythrocyte membrane. After the sample is confirmed as human blood, the next step is to identify the blood type or DNA with other technologies.

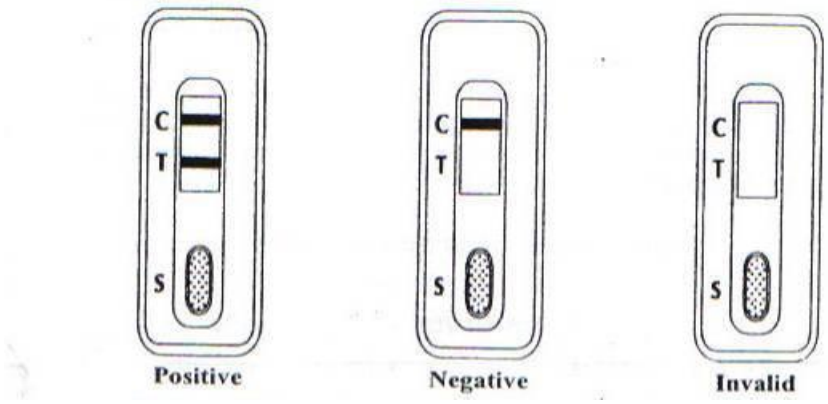


Figure 1-11 The immunochromatographic test

The first sample on the leftmost shows a positive result of blood by using immunochromatographic test. The first band is the control line that validates the test. The appearance of the second band indicates a positive result. The absence of the second band in the second sample represents a negative result. No band found in the third sample indicates the result is invalid.

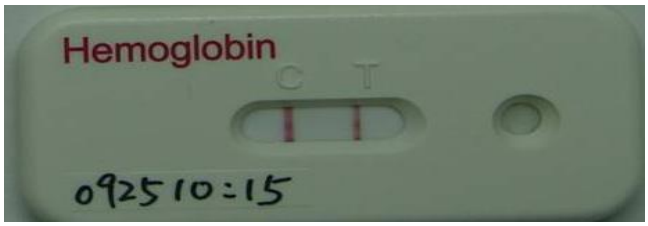


Figure 1-12 A commercial kit for blood immunochromatographic test

A positive result of a commercial kit for blood test. Two bands are found indicating the test is valid and positive.

1.1.2 Semen

Semen is another common body fluid found at crime scenes, especially in sexual assault cases. Semen exists in liquid form inside male body. It becomes jelly-like immediately after ejaculation, and returns to the liquid form 5 minutes later.

Besides spermatozoa (5%), the composition of human semen includes seminal plasma that is made up of complicated organic and inorganic components secreted by testes, epididymal ducts, seminal vesicles, and urethral glands [36]. The sperm concentration is about 20 million or more cells/ml [37]. Seminal plasma offers

protection and nutrition for spermatozoa. It can adjust the pH of female's vagina, making the environment less acidic for spermatozoa to survive and move around. Semen also contains fructose which is the energy source enabling the sperm to swim. There is a special odour of semen due to the presence of amines, including putrescine, spermine, and spermidine [38].

There is faint blue fluorescence to be seen if semen stain is placed under UV light with the 250-365nm wavelength. However, most fabrics nowadays are treated with fluorescent agents which can create a strong fluorescent background and interfere the observation of fluorescent emission from the semen left on the clothing. The basic confirmatory test for semen is through microscopic observation (Figure 1-13).

Sperm cells should be stained to enhance the contrast in the microscopy image and several different chemicals have been used for this purpose. For instance, haematoxylin and eosin stain (H&E stain) is widely used in the confirmation for the presence of semen [39]. As its name suggests, haematoxylin and eosin stain combines two dyes - haematoxylin and eosin, which were independently introduced by Böhmer in 1865 and by Fischer *et al.* in 1876, respectively [40]. The use of the combination of the two dyes was then firstly described by Wissowzky in 1875 [41]. Over a century later, H&E stain still remains as the most common staining method performed in histology histopathology laboratories and widely used in medical diagnosis. Haematoxylin is a dark blue dye and is cationic when it is in complex with aluminium salt which acts as a mordant to help haematoxylin to stain the tissues. As a result, haematoxylin can react with basophilic substances such as nucleic acids which are negatively charged and stain them dark blue or violet. On the other hand, eosin is a red dye which acts as an acidic dye. It can react with proteins containing amino acid

residues (such as arginine and lysine) and stain them dark red or pink. The uses in a combination of the two dyes make the stained tissue colour-contrasting so that different cell parts can be distinguished from one another. However, several reports reveal that H&E stain may result in DNA degradation [42, 43]. This should be noticed if the stained cells are harvested for subsequent PCR [44].

Besides, some laboratories have used Christmas tree stain for forensic routine examination. Sperm heads can be stained in red and the tails can be stained in green by Christmas tree stain [45] and it is also useful for microscopic examination of Spermatozoa for forensic scientists. Another staining method is utilizing alkaline fuchsin [46]. Nevertheless, Allery *et al.* reported that it is ineffective to detect spermatozoa with alkaline fuchsin stain whereas Christmas tree stain seems to be equivalent to H&E stain [45]. Visualization of stained sperm cells confirms the presence of semen immediately. However, sperm tails are fragile and will break down first. Hence it may not appear in semen stains. Spotting a sperm without its tail is still considered as a positive confirmation for the presence of semen. Besides, visual absence of sperm cells may result from the dilution of semen, the aging of the semen stain, oligospermia, azoospermia or sterilization. Therefore, a positive result from sperm analysis can confirm the presence of sperm while a negative result cannot disconfirm it.

Same as blood identification, there are presumptive and confirmatory tests for semen identification. Acid phosphatase (ACP) test is the most popular method among the presumptive tests [47] and it is used in both semen stain search and presumptive identification. ACP is an enzyme secreted by prostate gland. The concentration of ACP in human semen is much higher than in other body fluids, which makes it a good indicator for the presence of semen. The principle of ACP colour test is based on

phosphoserine hydrolysis by ACP and the colour visualization from interaction of its product with diazonium (Figure 1-14). Development of a purple colour within 1 minute represents a positive result (Figure 1-15). Unfortunately, some other body fluids may lead to false positives as well. Moreover, the ACP test is highly presumptive because vaginal secretions may also contain detectable levels of this enzyme [4]. In addition, analysis of old semen stains (more than 20 years) may take more than 1 minute to develop a purple colour.

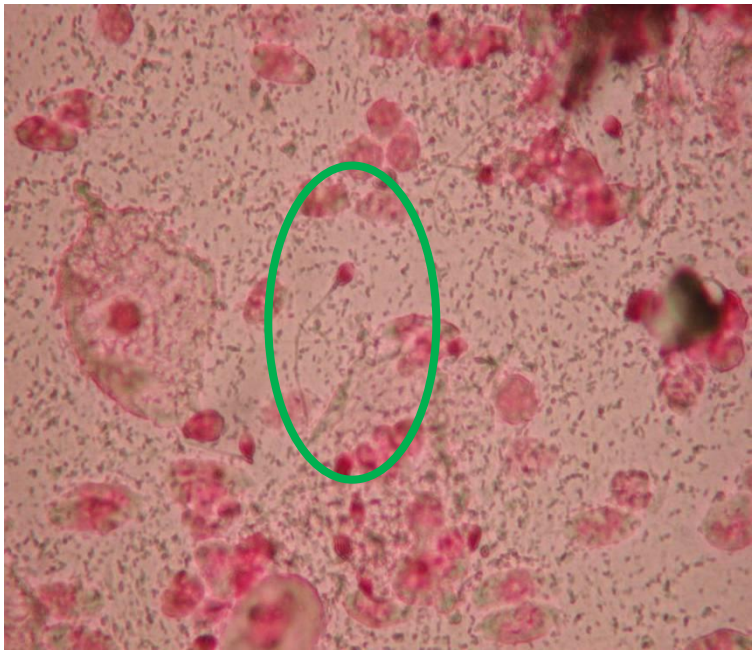


Figure 1-13 Image of sperms stained with Christmas tree stain under a microscope

The morphology of spermatozoa is very different from other cells; therefore, observation of spermatozoa is considered to be a positive identification of semen. The tails of sperms are usually degraded after ejaculation, so only the heads can be observed.

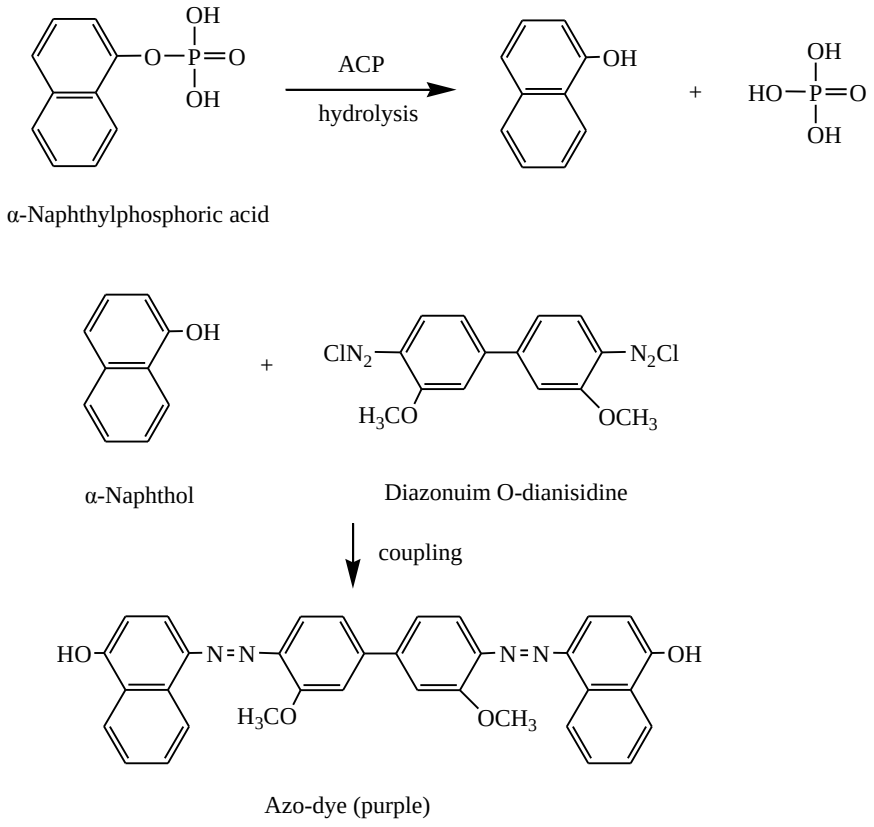


Figure 1-14 The structural formula and reaction of ACP test

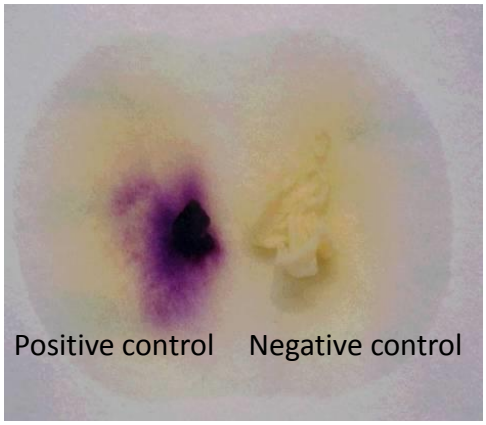


Figure 1-15 The ACP test in positive and negative control

The sample on the left shows a positive result of semen on a tissue (around 20µl). The sample on the right shows a negative result.

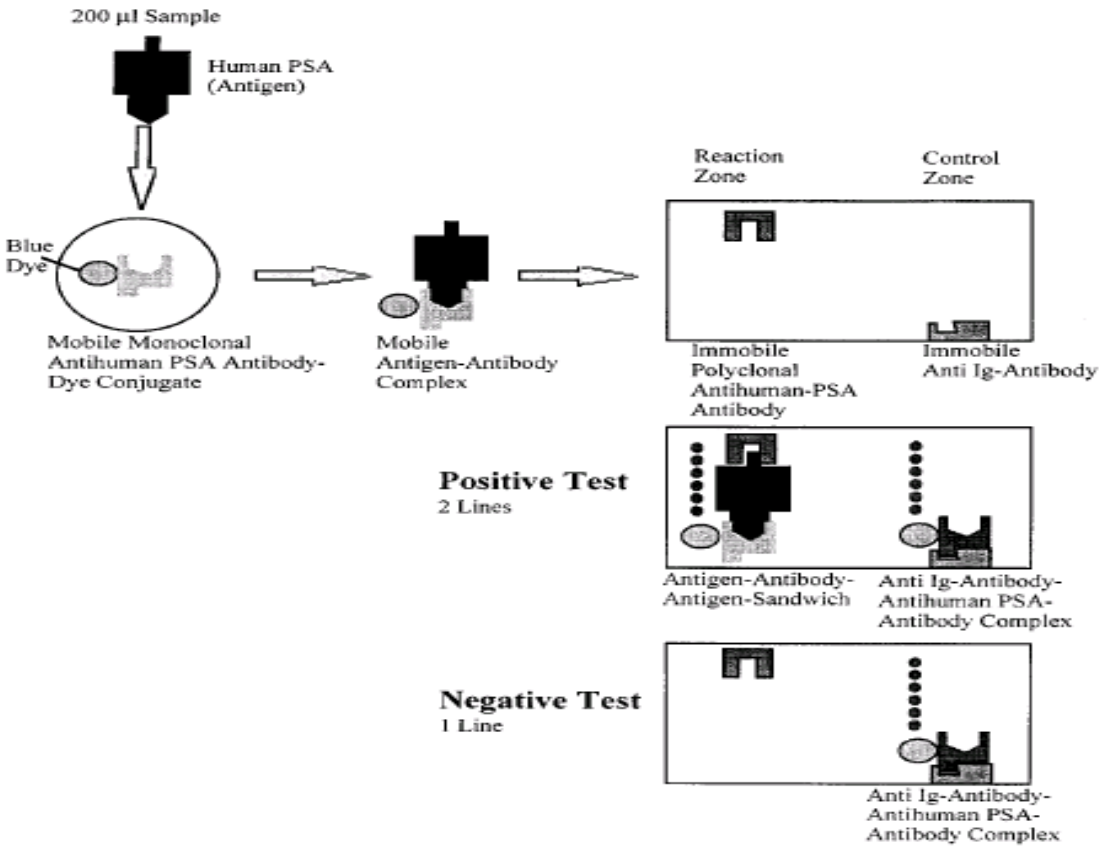


Figure 1-16 Principle of PSA membrane test assays

Adapted from Barni, F., et al., Forensic application of the luminol reaction as a presumptive test for latent blood detection. Talanta, 2007. 72(3): p. 896-913. For positive samples, target antigen (human PSA) will form a complex with mobile monoclonal antibody (anti-human PSA) with dye conjugate. The entire complex moves and binds to the reaction zone, resulting the formation of a band in this zone. The unused antibody-dye conjugate will then move on and bind to the control region.

Prostate-specific antigen (PSA, also known as p30) is a semen-specific glycoprotein secreted by prostate gland. The range of PSA concentration in seminal plasma is known to be 0.2 – 5.5 mg/ml [48], and the amount of PSA found in female vaginal secretion is at relatively low level [49, 50]. Therefore, it has been characterized as a useful marker for the presumptive test of semen in forensic science. PSA can be detected by electrophoresis or immunoassays (Figure 1-16) [48, 51, 52] and there are commercial kits available (Figure 1-17).

Positive control Negative control

Figure 1-17 A commercial kit for semen immunochromatographic test

The first sample is a positive result of a commercial kit for semen test. The formation of two bands indicates a valid and positive result. The second sample shows a negative result with only one band in the control region.

Semen samples from vasectomized, oligospermic, and azoospermic males may affect the result of semen identification because spermatozoa might not be observed in these samples by the microscope. The prevalence of azoospermia is approximately 1% [53] among all males and it is found in up to 10% to 20% of the men who present to an infertility clinic [54]. The frequency of contraceptive vasectomy in the world varies significantly with region and country. In developing countries, the overall prevalence of vasectomy is 2.5% whereas the prevalence exceeds 10% in 8 countries, including Australia, Bhutan, Canada, the Netherlands, New Zealand, the Republic of Korea, Great Britain, and the United States [55]. Although spermatozoa may not be present in the semen of vasectomized or azoospermic males, these males can still produce normal amounts of semen containing both prostate gland and seminal vesicle secretions which are detectable by other forensic laboratory tests [48, 56]. Thus, ACP and PSA still can be used as indications for the semen samples from these males [52, 57]. However, some studies have shown that PSA can also exist in female urine [58, 59], breast milk [60], and sweat gland [61]. Hence, additional caution is always required when interpreting a positive result from PSA as it may result in false positive. Nevertheless, PSA test is still a useful method for the identification of semen.

1.1.3 Saliva

The primary component of human saliva is water (more than 98%) and the other 2% is composed of electrolytes, mucus, antibacterial substances, enzymes, and epithelial cells. Saliva helps to moisten food, making it easier to swallow; it also contains amylase enzyme to break down starch. Saliva is easily left on food containers or drinking glasses. Thus, saliva evidence can often be found on the edge of a cup, the rim of a bottle, on a straw or tableware at crime scenes. It can also be found from bite marks [62, 63] even if the body is submerged in water [64, 65].

Identification of saliva is very important in some cases, especially in sexual assault cases involving oral intercourse. Besides, at the crime scene, the suspect may leave some items, such as cigarette butts, chewing gum, and betel nut, which could provide sufficient saliva for DNA analysis. Saliva stain is detectable by the naked eye by using UV light. It is in a lower intensity compared to semen [66]. Although saliva can be detected by forensic light source (FLS) with goggles/filters (for example, excitation wavelength of 450nm with orange goggles or 555nm interference filters) [67], the positive detection is not specific to saliva.

Traditionally, the presumptive test of saliva is performed by using radial diffusion in a starch/agarose gel [68] but it lacks sensitivity and specificity. Another method currently used for amylase detection by forensic scientists is Phadebas® forensic saliva test [69]. It utilises a water-insoluble substrate which cross-linked starch polymer carrying a blue dye. The substrate is hydrolysed on the presence of amylase and forms water-soluble blue fragments. Thus, the presumptive test can be evaluated by the observation of the blue dye and comparison between positive and

negative control [70]. The commercial products are divided into two categories: the tube test and the press test. The tube test is used for presumptive test of a single stain and is more sensitive than the press test. A small portion of suspicious stain and the required amount of Phadebas® chemical with sterile distilled water are mixed in a tube and then incubated at 37°C for 30 minutes. After centrifuging at 10,000g for 1 minute, a positive amylase reaction will produce a blue coloured supernatant solution. Both positive and negative control samples are required for the tests (Figure 1-18). The press test is used for detecting and identifying hidden saliva stains. It utilises a filter paper which has been pre-treated with the Phadebas® chemical [69]. The paper is moistened before the test and then pressed against an alleged saliva stain. The saliva deposits can be located with a diffuse blue colour. Figure 1-19 illustrates the progress of the press test.

Another similar method using red-starch paper is available as well [71]. Also, several enzyme-linked immunosorbent assay (ELISA) commercial kits (Figure 1-20) are ready for forensic usage in detecting saliva [72]. More detailed introduction to saliva is addressed in Chapter 4.

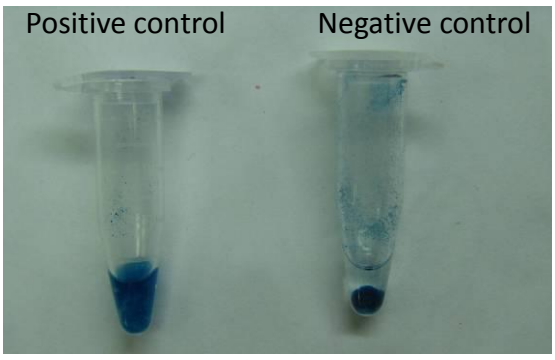


Figure 1-18 Amylase tube test in positive and negative control

Positive and negative results of Phadebas® Amylase test [70]. In the left tube (positive control), amylase in the sample reacts with the homogeneously interlinked starch polymers in the tablet and produces a water soluble free dye molecule of a blue colour. In the negative control, the starch polymers are not broken down and the supernatant is clear.

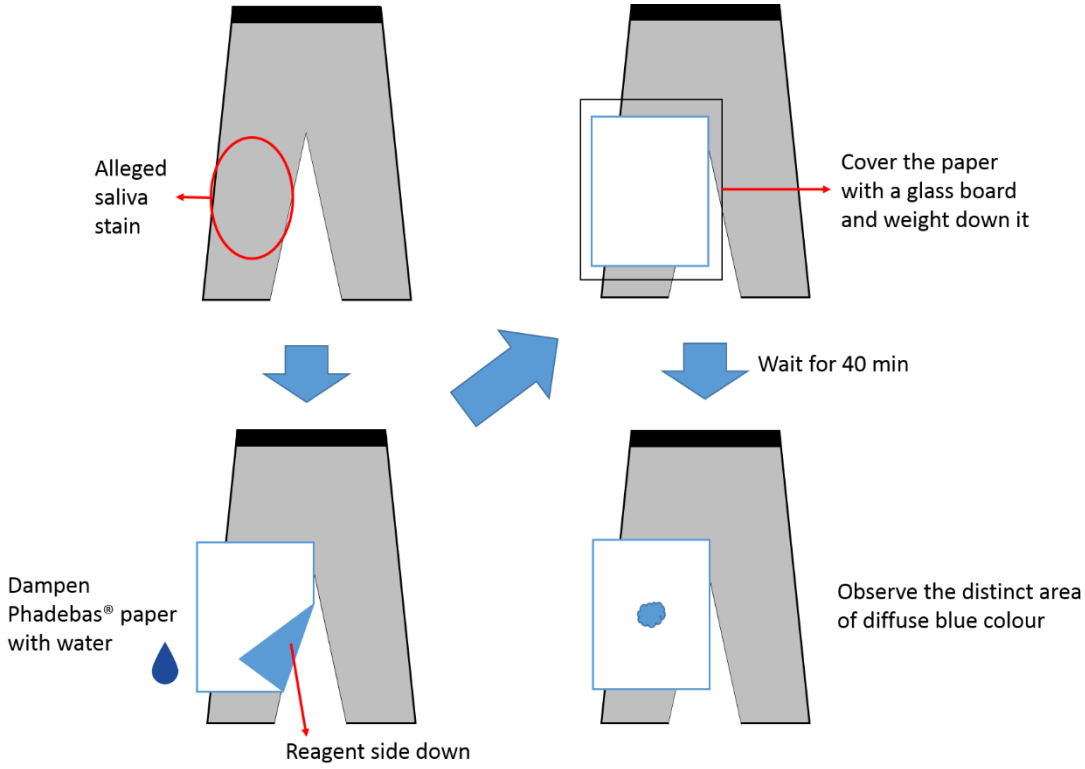


Figure 1-19 An illustration of the Phadebas® press test

The item to be tested is placed on a flat surface and then the press paper is placed over the area, with the blue reagent side facing to the item. After dampening the paper, cover the paper with a clean glass board and place a weight on top of the glass to ensure good contact between the item and the paper. A positive is identified as a distinct area of diffuse blue colour on the non-reagent side of the paper.

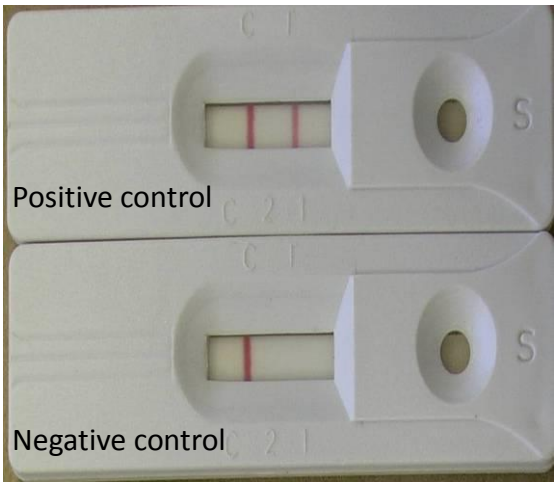


Figure 1-20 The result of a positive and a negative saliva control sample tested by the commercial kit

Positive and negative results of a commercial test kit for saliva. Two bands can be seen on the card which shows it is valid and positive. The second sample is a negative result which shows only one band in the control region.

1.1.4 Menstrual blood

The menstrual cycle is a series of regular changes that optimize a woman's body for pregnancy in the uterus and ovaries [73]. The cycle is controlled by hormonal changes. Basically, the menstrual cycle can be divided into 4 main phases (Figure 1-21), including menstrual phase (from day 1 to 5), follicular phase (from day 6 to 13), ovulation phase (around day 14), and luteal phase (from day 15 to 28). Nevertheless, the length of the menstrual cycle varies from woman to woman [74]. In menstrual phase, the drop in hormone levels causes the shedding of the thickened uterine lining and bleeding. Most women menstruate for 2 to 7 days and lose between 20 and 80 millilitres of blood during this period. Follicular phase begins on the first day of menstruation. After the bleeding, the raising of hormone levels stimulates the ovarian follicles and makes it ready to release an egg. During the maturation, the egg follicle secretes a hormone which makes the endometrium start to replenish itself from a layer of stem cells in the wall of the uterus. The matured egg cell is released during ovulation phase and travels down the fallopian tube to the uterus over the next few days. In luteal phase, progesterone level increases and helps prepare the uterine lining for pregnancy. If the egg is not fertilized in this phase, it disintegrates. Then hormone levels drop again and cause the menstrual phase of the next cycle. The whole period lasts around 21 to 35 days.

Menstrual fluid is composed of a mixture of blood (nearly half), desquamated endometrial tissue [75], sloughed vaginal epithelial cells, cervico-vaginal secretions, and endogenous vaginal microbes [76]. Thus, it contains the composition of blood and vaginal secretion. The composition varies according to the phases of menstruation

which start with an opaque liquid stage, followed by a thicker stage and then a clear liquid stage.

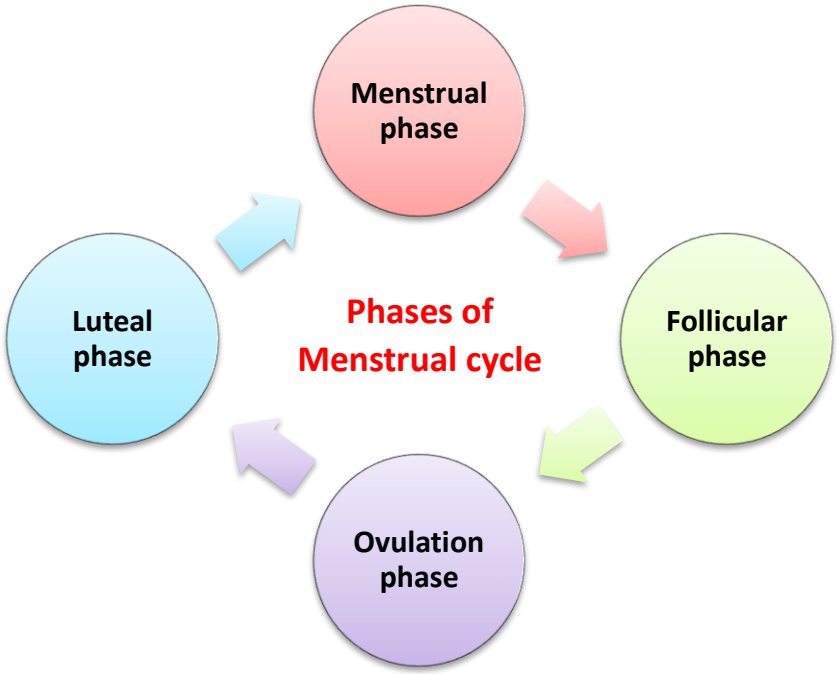


Figure 1-21 An illustration of different phases of the menstrual cycle

Comparing with venous blood, menstrual blood is darker and thicker [77]. Besides, menstrual blood differs from venous blood in the composition, including haematological components (coagulation factors), inorganic materials, and organic materials [76]. For example, pro-thrombin and free thrombin can be detected in venous blood, but they are absent from menstrual fluid [78]. On the contrary, plasmin activity is positive in menstrual fluid but not found in venous blood [79]. The composition and physical properties of menstrual blood vary among individuals. In some sexual assault cases, identification of menstrual blood is crucial in some special scenarios. For example, the victim claimed that she was bleeding because of sexual violation. On the other hand, the suspect declared himself innocent and asserted that

the blood is menstrual blood. In this situation, identification of the type of blood is crucial to the investigation.

Menstrual blood was identified early on by checking epithelial cells of the endometrium, cervix, or vagina via microscope. Masumi Shiraishi *et al.* used the "Fibrin-plate method" to confirm the presence of menstrual blood by observing the fibrinolytic activity [80]. It could also be identified by using a haemagglutination inhibition test [81]. Minoru Asano *et al.* found that lactate dehydrogenase 4 (LDH-4) and lactate dehydrogenase 5 (LDH-5) are highly expressed in menstrual blood and can be used as markers for the identification of menstrual blood by electrophoresis in alternative [82].

1.1.5 Vaginal secretion

Vaginal secretion contains the secretions made by glands inside the vagina and cervix. It could carry away dead cells and bacteria to keep the vagina clean and prevent it from infections. Vaginal secretion may also contain menstrual blood when it is collected from the female during her menstrual period. The amount, colour and odour of the vaginal secretion can vary depending on different conditions, including the menstrual cycle and sexually transmitted diseases [83], so the features of the vaginal secretion can be a symptom of reflection referring to the different stages of the menstrual cycle, infection or disease. Some materials or physical conditions may affect the features of the vaginal secretions as well, including contraceptive drugs, antibiotics, allergy, pregnancy, childbirth, malnutrition or even stress.

Although the vaginal secretion is not as common at crime scenes as blood, semen or saliva, sometimes it is important to confirm whether or not the vaginal

secretion is present on objects from the crime scene. For example, in some sexual assault cases with a foreign object penetration into the vagina, the identification of vaginal secretion transferred from the victim to the object can be crucial to the criminal investigation.

Most forensically relevant body fluids can be identified by conventional immunological and/or enzymatic tests such as the methods described previously. For vaginal secretion, some preliminary screen methods have been demonstrated. Sikirzhytskaya *et al.* represented Raman spectroscopic signature of vaginal secretion and its potential application to forensic identification of body fluids [84]. Besides, Lugol's iodine staining technique is used for the detection of vaginal epithelial cells [85]. The method relies on the principle that iodine reacts with intracellular glycogen to produce a dark brown colour. However, Lugol's method can no longer be assumed to prove the presence of vaginal cells due to its high error rate [85]. There are still some other methods for vaginal secretion identification, but none of them is conclusive for identifying vaginal secretion [3, 86]. Recently, forensic scientists have turned to seek molecular identification of vaginal secretion by microbial signature [86-88]. For example, a woman's vagina is kept healthy by *Lactobacilli* [89], so relative quantification of *Lactobacillus* DNA can be a simple identification method for vaginal secretion and this may provide a new promising tool.

1.1.6 Sweat

Regarding the identification of body fluids, sweat has been considered as one of the body fluids that are most difficult to be found and identified. It is usually left at the crime scene such as on the weapon, a facial tissue, a glove, a hat or even in a

fingerprint. Sweat is usually ignored by the forensic investigators at crime scenes since it is difficult to find and no confirmatory test for the identification of sweat has been developed yet. Although no application of sweat identification is practically used to criminal investigation so far, yet it might be applicable in the future and may play a role in forensic science and benefit criminal investigation. For example, DNA profiles can be obtained from trace biological evidence such as fingerprints which may contain sweat. Hence, identifying sweat can be important for analysing these types of evidence [15].

The main constituent of sweat is water (98% to 99%) and the other 1% to 2% is composed of minerals, urea, lactate acid and fatty acids. The mineral composition may vary with individuals, the environment and the physical condition [90, 91]. Although no confirmatory test has yet been developed for the identification of sweat, several attempts have been made. Some presumptive tests are used for sweat, including the detection of urea and lactic acid [92] and observation of skin cells under microscopes [93]. Sikirzhytski *et al.* represented a potential nondestructive identification of traces of sweat by using multidimensional Roman spectroscopic signature [94]. Sagawa *et al.* found the production and characterization of a monoclonal antibody for sweat-specific protein and its application to sweat identification [95]. Schittek *et al.* demonstrated an antimicrobial protein called dermcidin (*DCD*) which is specifically and constitutively expressed in the sweat glands. *DCD* is mixed with sweat and transported to the surface of the skin [96]. *DCD* is processed into 47 peptides with antimicrobial activity against pathogens like *Escherichia. coli*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Candida albicans*. Koichi Sakurada *et al*. reported the detection of *DCD* for sweat identification by real-time RT-PCR and ELISA [93]. Kazunori Sagawa *et al*. found that a monoclonal antibody

(G-81) is stable and able to detect sweat stains and suggested it as one of the markers for the identification of sweat [95].

1.1.7 Urine

Urine is a mixture with more than 95% water and 3-5% organic as well as inorganic compounds. These compounds mainly include urea, chloride, sodium and potassium ions, creatinine and some proteins, such as albumin and Tamm-Horsfall protein. Urine will fluoresce when exposed to ultraviolet light and can be distinguished by its distinctive odour due to the release of ammonia from the breakdown of urea after the urine is excreted. However, ammonia can also result from decomposition of animal excreta or decay of organisms, which may interfere the identification of urine at a crime scene. There are several presumptive tests for the identification of urine. One of the most popular presumptive test methods is using para-dimethylaminocinnamaldehyde (DMAC) to react with urea, which is a simple, rapid and safe test [97]. Unfortunately, the DMAC test is not specific to urine and may yield false positive result in some other body fluids. Urine can also be detected by estimating creatinine with the modified Jaffe colour test. Creatinine reacts with picric acid in an alkaline solution and forms a reddish-orange colour [98, 99]. Besides, Tamm-Horsfall protein and Uroplakin III are also evaluated for forensic identification of urine [100, 101]. A non-destructive confirmatory identification of urine by SEM-EDX is also revealed by identifying the relative concentrations of sodium, phosphorus, sulfur, chlorine, potassium, calcium, and other metal trace elements in the urine sample [3].

Urine identification can be useful in some special scenarios. For example, the confirmation of the presence of urine could be useful for an alleged sexual assault or

violent crime involving urination. Urine identification can be also useful when the prosecutor wants to confirm the urine sample provided by the drug addict for drug inspection is not a swap of other liquids than urine. Besides, strangulation victims might excrete urine involuntarily prior to death [15]. In such an incident, the identification and location of urine might provide where the first scene is.

1.1.8 Other body fluids

Beside the body fluids mentioned above, several biological fluids may be found at the crime scene. Although they are less frequently encountered, they may require forensic analysis in some special scenarios. Here faeces, vomitus, and breast milk were introduced.

Faeces are metabolic waste matters which result from the digestion of food by the gastrointestinal tract and are discharged through the cloaca. Human faeces contain water, undigested food particles, sloughed epithelial cells, intestinal bacteria and so on. Faecal samples can be important to sexual assault cases in which anal intercourse occurs. Undigested or partially digested plant cells in faeces can be analysed by microscopic examination [102]. Besides, Lloyd *et al.* represented a spectrometric study on the fluorescence detection of faecal urobilinoids [103].

Vomiting can be induced by an uncontrollable reflex resulting from a wide range of factors or forced by irritating the throat. Vomitus analysis can be used for crime scene reconstruction in an affray. Vomitus can also be identified by microscopic examination for the undigested plant cells [102]. Yamada *et al.* revealed another method for vomitus identification by pepsin assay utilizing fibrin blue-agarose gel plate [104].

Identification of breast milk can be helpful in some special sexual assault cases involving a breast-feeding woman. Yamawaki *et al.* studied the macronutrient, mineral and trace element composition of breast milk [105]. However, no useful method for identification of breast milk has been proposed so far. Besides, breast milk may cause false positives in some presumptive tests of body fluids, such as the amylase test for saliva [3].

1.2 DNA analysis in forensic science

DNA usually can be obtained from biological samples. DNA analysis contributes a lot to forensic science and criminal investigation via DNA profiling. Forensic DNA analysis was first developed by Alec Jeffrey in 1985, who utilised Restriction Fragment Length Polymorphism (RFLP) for DNA fingerprinting [106, 107]. However, the reliability of DNA evidence, stability of identifying methods, and development of statistical methods raised a lot of problems in the beginning. Over the years, short tandem repeats (STR) analysis has been well-developed and widely used in forensic DNA profiling. This method targets highly polymorphic regions that contain 2-8 bases repeated sequences of DNA [108]. STRs can be found in or between genes and some of them are associated with the expressions or functions of genes. Some diseases also have been confirmed to be associated with STRs [109, 110]. Comparing with the other sequences in the genome, STRs are relatively unstable with mutation rate of around 0.1% during meiosis [111, 112]. The specific target regions of DNA can be amplified by polymerase chain reaction (PCR) and then the PCR products can be separated and detected by electrophoresis. Each individual has a specific allele indicated by the length of DNA fragment. A DNA profile can be built with a number of different STRs

and further used to compare with other profiles in criminal investigation. For example, DNA-STR profiles from a victim and an exhibition can be compared to each other. If it is a match, then the victim would be strongly considered as the donor of this DNA from the exhibition. A following calculation of probability then will be applied to the match. On the other hand, the victim will be excluded as the donor if his/her profile does not match the profile of the exhibition.

Different commercial kits are available for different STR-based DNA-profiling systems [113-115]. No matter what system is used, the comparison of DNA profiles is based on statistics. Based on the assumption of Hardy–Weinberg principle [116], the random match probability (RMP) of a genotype from a locus can be calculated with a proper and valid population data. As most of the STR loci used for DNA profiling are independently assorted, the product of random match probability can be applied to estimate the rarity of a DNA-STR profile. If the DNA profiles obtained from an individual and a sample are the same (a match), the sample might either come from the suspect or a random person who happens to have the same DNA profile. Then the RMP could be used to evaluate the possibility in the latter situation. If the DNA profile is very rare (the RMP is relatively small), the person might be related to the sample. On the contrary, the person might be unrelated to the crime and the match occurs by chance if the DNA profile is common. Analysing more STR loci could increase the power of discrimination. DNA databases based on STRs have been established in many countries around the world [117]. These databases are irreplaceable tools for searching suspects, victims and/or related evidences. Due to its high reliability, DNA profiling is now widely accepted by most countries all over the world. There are countless cases that courts accepted DNA as evidence in the trials and numerous offenders have been convicted by matching their DNA profiles with the DNA collected

at crime scenes. DNA profiles not only can be used to identify the origin of a DNA sample at a crime scene, but also can be used in paternity testing based on Mendel's laws.

1.3 RNA analysis in forensic science

Unlike DNA analysis, RNA analysis is relatively new in forensic science. RNA analysis had been ignored in the past while DNA technology had been widely used in forensic science because RNA is alleged not only to decay rapidly post-mortem and in vitro but also to be contaminated easily. Besides, inherent degradation may occur to mRNA. For example, nonsense-mediated mRNA decay (NMD) is a surveillance pathway existing in all eukaryotes to reduce errors from gene expression by selective degradation of mRNA transcripts [118, 119]. It was not until Oehmichen *et al.* proposed post-mortem DNA and RNA synthesis from cadavers in 1984 [120] did the application of RNA analysis to forensic science launch. With the development of RT-PCR, more and more forensic studies have focused on RNA analysis and lots of reports regarding the potential of RNA technologies have been published by forensic scientists [7]. Several attempts of potential applications by utilizing RNA analysis in forensic science have been made. For example, several mRNA markers have been revealed concerning their potential for discrimination among several body fluids. These mRNA markers can be used for identification of the forensically most relevant body fluids.

1.3.1 Introduction to RNA

Like DNA, RNA is also a nucleic acid and very similar to DNA, but there are some important structural differences between them. First of all, RNA is more often found in nature as a single-stranded molecule which may fold onto itself, rather than a double-stranded molecule like DNA. Next, unlike DNA which is only found in the cell nucleus, RNA is mostly found in the cytoplasm. Furthermore, the complementary base to adenine in RNA is uracil (a demethylated form of thymine) whereas it is thymine in DNA.

Typically, a mammalian cell contains 10 to 30pg of total RNA [121]. Several different forms of RNA molecules are known which can be differentiated by their specific configuration, structure, and function. Basically, there are three types of RNA, including messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). Besides, several kinds of RNAs involving in the regulation of the gene expression, such as microRNA, are also found in the cell. During the transcription process, information on DNA is copied into mRNA which is then translated into proteins. Different types of cells consist of different proteins, and therefore, identifying corresponding mRNA of a specific protein allows the identification of the cell type. RNA may contain secondary structures which make a better stability. However, RNA is still less stable than DNA due to several reasons. First of all, DNA contains deoxyribose while RNA contains ribose where there is a hydroxyl bond (-OH) attached. The ribose sugar in RNA is more reactive than deoxyribose sugar in DNA. Therefore, the hydroxyl bond makes RNA less stable because it is prone to hydrolysis [122]. Besides, double-stranded DNA has relatively small grooves which enzymes cannot attach to, so enzymes cannot decompose DNA easily. In addition, it requires less energy to break down RNA than

DNA [123]. Besides, RNA is relatively less stable than DNA and cannot be preserved for a long time due to ubiquitous presence of ribonuclease enzymes [7].

However, some studies demonstrated that stable mRNA may be obtained from post-mortem tissues with long autolysis time [124, 125], especially in dried or frozen samples, where mRNA degradation is slower. Other studies showed that it is possible to detect mRNA markers in old body fluid stains dating back up to 16-23 years [126, 127]. The analysis of cell-specific mRNA expression has been used for the identification of body fluids in forensic science [128]. In this present study, analysis of mRNA was applied to identify different body fluids with 18S rRNA as an internal control for the identification and evaluate the time since deposition of biological materials.

1.3.1.1 Messenger RNA

Based on the central dogma of molecular biology (Figure 1-22) that describes the normal flow of biological information, there are 3 general transfers: (1) DNA can be copied to DNA (DNA replication), (2) DNA information can be copied into mRNA (transcription), and (3) proteins can be synthesized using the information in mRNA as a template (translation), as shown by the solid arrows in Figure 1-22 [129]. Reverse transcription is a special transfer of information from RNA to DNA. In the reverse transcription reaction, RNA is used as a template and transcribed into complementary DNA (cDNA) which is synthesized in the reaction from mature mRNA, catalysed by reverse transcriptase and DNA polymerase. Reverse transcriptase extracted from retrovirus is required in the reaction.

During transcription, RNA polymerase binds on the particular segment of DNA and makes a copy of a gene from DNA to mRNA. The major steps of transcription

include pre-initiation, initiation, promoter clearance, elongation, and termination. In eukaryotic organisms, the primary transcripts (also named pre-mRNAs) require further extensive processing of 5’ cap addition, polyadenylation, and splicing reactions in succession [130]. A 7-methylguanosine cap is added to the 5’ end of a pre-mRNA shortly while elongation is still in progress. The 5’ cap allows the ribosome to recognize the beginning of the mRNA and protects the pre-mRNA from degradation. On the other hand, a poly-A tail is added to the 3’ end of the pre-mRNA once after the elongation. The poly-A tail adds stability to the molecule and involves in binding proteins in initiating translation. Eukaryotes have regions of DNA within genes that are transcribed into RNA but not translated into proteins later. These regions, namely introns, are removed from pre-mRNA and spliced. After splicing, the mRNA is exported to the cytoplasm. Messenger RNA accounts for only 1% to 5% of total RNA [131]. There are approximately 360,000 mRNA molecules in a single cell, with approximately 20,000 to 30,000 different mRNA species whose copy numbers range from 15 to 12,000 depending on the species [132].

Not only translating into proteins, RNA also can be a template for DNA synthesis. All tumour viruses, such as the AIDS virus, can make an RNA-dependent DNA polymerase (reverse transcriptase) that synthesizes a single-stranded DNA complementary to the viral RNA. Reverse transcription polymerase chain reaction (RT-PCR) is widely used in research.

RNA was not considered useful to forensic science in the past. However, it has been revealed that RNA can be helpful in some fields, including identification of body fluids and ascertaining the time since deposition of evidence. Body fluids are common biological materials and it is getting important to identify the type of body fluids found at crime scenes. At present, most forensic laboratories use enzymatic tests to screen

body fluids before extracting DNA. Since these methods have drawbacks as mentioned above and RNA can be confirmatory, sensitive and specific to individual body fluid, RNA has been considered and tested for its potential to identify different body fluids.

RNA, especially mRNA, can play an important role in forensic identification. First of all, there are a lot of different mRNAs present in different kinds of tissues or body fluids. Some of them are tissue or fluid specific and can be used as markers to identify the samples. Instead of using traditional presumptive methods to detect and identify different body fluids, mRNA can be applied to identification of body fluids as well.

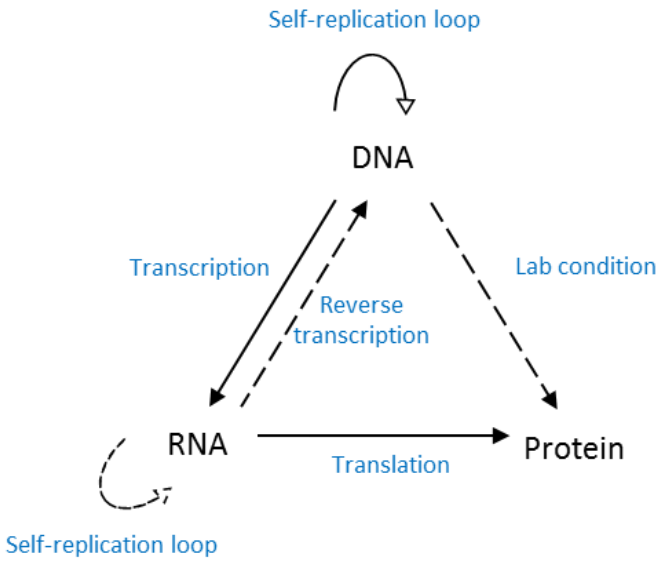


Figure 1-22 The central dogma of molecular biology

1.3.1.2 Ribosomal RNA

Ribosomal RNA comprises a major portion of ribosome which involves in synthesis of polypeptide chains. Prokaryotic ribosomes are composed of

approximately 65% of rRNA and 35% of ribosomal proteins by weight. Ribosomal RNA comprises about 80% of total RNA in a cell [133]. Since ribosome serves for protein synthesis, it is essential in all living organisms. Both prokaryotic and eukaryotic ribosomes can be divided into two subunits (Table 1-2). The S-values indicate how fast particles sediment through a particular solution in an ultracentrifuge. The value only gives a rough indication of their size and it is not linearly related to molecular weight [134]. Ribosomal RNA can be used in research of molecular biology. The *18S* and *28S* ribosomal subunits are used to evaluate the RNA quality for experiments by calculating their ratio [135]. Besides, the *18S* subunit is usually used as an internal standard marker for gene expression analysis. In this study, *18S* rRNA was used as an internal control marker.

Table 1-2 Ribosomal RNA subunits

Type	Size	Large subunit	Small subunit	Proteins
Prokaryotic	70S	50S (5S, 23S)	30S (16S)	52
Eukaryotic	80S	60S (5S, 5.8S, 28S)	40S (18S)	82

1.3.1.3 Transfer RNA and the other types of RNA

Transfer RNA (tRNA) is a kind of RNA molecule transcribed directly from DNA and its length varies from about 74 to 95 nucleotides. It serves as the decoder to translate an mRNA sequence into a protein. There are about 30 different tRNAs. Each of them can be attached with the amino acid that corresponds to the anticodon and recognizes a three-nucleotide sequence (codon) of mRNA through its own anticodon. The tRNAs attached with amino acids are then delivered to the ribosome. If the

anticodon of the tRNA matches the codon of the mRNA sequence, the attached amino acid will be transferred to the growing polypeptide chain to synthesise the protein which is catalysed by the ribosome. In forensic genetics, Vennemann *et al.* described the possible application to quantify gene expression levels of specific genes, including tRNA [136]. Nonetheless, most studies focused on mRNA and rRNA instead of tRNA.

A group of relatively short RNAs also involve in the regulation of gene expression, such as microRNAs (miRNAs), anti-sense RNAs (asRNAs), or small interfering RNAs (siRNAs). Among these short RNAs, microRNAs have been applied to forensic science. MicroRNAs (miRNAs) are small single-stranded RNA molecules which only contain 21-24 nucleotides in length. It is now known that miRNAs play an important role in gene regulation and are related to several diseases, such as Alzheimer's disease[137] and colorectal cancer[138]. Several studies showed the relative abundance of miRNAs in human tissues, with numerous miRNAs reported to be tissue-specific. Recently, some studies showed miRNA expression in forensically relevant, dried biological fluids [139]. Park *et al.* screened and evaluated microRNA markers for forensic body fluid identification by microarray and qRT-PCR [140]. Wang *et al.* also presented a model for data analysis of microRNA expression in forensic body fluid identification [5], which showed that the property of tissue-specific expression enables miRNA as an ideal biomarker for body fluid identification.

1.3.2 Cell-specific (m)RNA expression

The human genome encodes approximately 20000–25000 protein-coding genes which account for about 1.5% of the genome [141]. Some of the genes maintain constant expression levels in any type of cells as the proteins produced by these genes

often involve in basic cell maintenance. These genes are also named housekeeping genes [142]. On the other hand, some genes are expressed as part of the process of cell differentiation or as a result of cell differentiation. Thus, there are unique patterns of gene expression for each cell type or tissue [143]. As different types of cells need to manufacture their own specific proteins, gene regulation is required. It includes a wide range of mechanisms that enhance or reduce the production of specific proteins or RNA. Multicellular eukaryotes control cell differentiation through complex, precise, temporal, and spatial regulation of gene expression. The phenomenon of cell-specific differences of multicellular organisms reveals the possibility of identifying body fluids by specifically expressed mRNA. Several specific mRNA markers have been evaluated for different biological materials (Table 1-3).

Table 1-3 Body fluid-specific mRNA markers for different biological materials

Target	Marker	Gene name	Amplicon size (bp)	Reference
Blood	*HBB*	Haemoglobin subunit beta	61	Haas *et al.* [128]
	SPTB	Spectrin, beta	247	Juusola and Ballantyne [144]
	PBGD	Porphobilinogen deaminase	177	Gubin and Miller [145]
	Glyco A	Glycophorin A	115	Roeder and Haas [146]
	ALAS2	Delta-aminolevulinate synthase	136	Haas *et al.* [147]
	PRF1	Perforin	102	Roeder and Haas [146]
	PF4	Platelet factor 4	124	Roeder and Haas [146]
	CD93	Cluster of Differentiation 93	151	Lindenbergh et al. [148]
	AMICA1	adhesion molecule (interacts with CXADR antigen 1)	136	Lindenbergh et al. [148]
Spermatozoa	*PRM1*	Protamine 1	153	Steger *et al.* [149]
	PRM2	Protamine 2	294	Steger *et al.* [149]
Semen	*TGM4*	Transglutaminase 4	215	Fang *et al.* [150]
	KLK3	kallikrein-related peptidase 3	100	Roeder and Haas [146]
	SEMG1	Semenogelin 1	158	Roeder and Haas [146]
Saliva	*STATH*	Statherin	198	Gubin and Miller [145]
	HTN3	Histatin 3	134	Gubin and Miller [145]
	SMR3B	Submaxillary gland androgen regulated protein 3B	147	Roeder and Haas [146]
	PRB4	Proline-rich protein BstN1 subfamily 4	160	Roeder and Haas [146]
	MUC7	Mucin 7	197	Roeder and Haas [146]

Table 1-3 (continued)

Target	Marker	Gene name	Amplicon size (bp)	Reference
Oral mucosa	*KRT4*	Keratin 4	81	Lindenbergh et al. [148]
	SPRR2A	Small proline-rich protein 2A	106	Lindenbergh et al. [148]
	KRT13	Keratin 13	131	Lindenbergh et al. [148]
Menstrual blood	*MMP7*	Metallopeptidase 7	240	Juusola and Ballantyne [144]
	MMP10	Metallopeptidase 10	143	Roeder and Haas [146]
	MMP11	Metallopeptidase 11	173	Bauer [151]
	MSX1	Msh homeobox 1	96	Roeder and Haas [146]
	SFRP4	Secreted frizzled-related protein 4	136	Roeder and Haas [146]
	LEFTY2	Left-right determination factor 2	130	Roeder and Haas [146]
Vaginal secretion	*HBD1*	Beta defensin 1	213	Juusola and Ballantyne [144]
	MUC4	Mucin 4	235	Juusola and Ballantyne [144]
	Lgas	16S-23S intergenic spacer region (*Lactobacillus. gasseri*, vaginal-specific bacteria)	279	Fleming and Harbison [87]
	Lcris	16S-23S intergenic spacer region (*Lactobacillus. crispatus*, vaginal-specific bacteria)	253	Fleming and Harbison [87]
Skin	*CDSN*	Corneodesmosin	79	Commercially available
	LOR	Loricrin	105	Commercially available
	KRT9	Keratin 9	94	Commercially available
	ACTB	Beta actin	74	[152]

Housekeeping genes, such as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *18S rRNA* [148], transcription elongation factor 1α (*TEF*), glucose 6-phosphate dehydrogenase (*G6PDH*), and ubiquitin conjugating enzyme (*UCE*), have been used widely as internal controls in experimental expression studies [146, 153, 154]. Several multiplex systems have been established for identification of biological materials [148, 155, 156]. To investigate more markers, Park *et al.* systematically evaluated the body fluid-specific expression of mRNA and presented novel mRNA markers for identification of forensic body fluids by performing DNA microarray [157]. Besides, microarray screening and qRT-PCR are also used for the evaluation of microRNA markers [140].

1.3.3 Sources of RNA from different body fluids

The main source of RNA varies from body fluids to body fluids. Blood contains many RNA-containing blood cells (leukocytes and reticulocytes) which can be valuable sources of RNA for blood [158]. Moreover, red blood cells and platelets still contain a minute amount of translational active mRNAs which are transcribed in their early stages. These can also be the source of RNA from blood. In semen, mRNA can be obtained from sperm cells. Besides, epithelial cells shed from the surface of their related glands or ducts may be mixed in the related body fluid. Thus, RNA obtained from a body fluid may also come from the epithelial cells of the related glands or ducts.

Extracellular nucleic acids (a small amount of nucleic acids including DNA, mRNA, and microRNA fragments) were first detected in the 1940s when nucleic acids were identified in blood plasma of healthy individuals [159]. These cell-free nucleic acids also have been detected in many body fluids [160-163]. The potential sources of

extracellular nucleic acids are extracellular vesicles which are endogenous vesicular structures and contain proteins and nucleic acids secreted by most eukaryotic cells [164]. Apoptotic and necrotic cell death along with active cell secretion may result in circulating cell-free nucleic acids [163, 165, 166]. However, some researchers argue that and refer to the possibility of nucleic acid secretion by normal and tumour cells [167]. Individuals suffering from various clinical disorders (such as cancer) or physiological changes (such as pregnancy) may reduce or increase the levels of cell-free nucleic acids [168]. Thus, cell-free nucleic acids have been noticed on medical studies to monitor diseases by evaluating the level of these nucleic acids. The levels of cell-free nucleic acids have been found to be variable and subjective to factors other than medical conditions [169].

Apart from the above, RNA may come from micro-organisms or other materials in the body fluid. For example, the sources of salivary RNA include not only oral epithelial cells but also oral micro-organisms [170] or even remnants of food. RNA from blood can also reach the saliva from wounds in the oral cavity [171]. As for menstrual blood, it contains blood and usually mixes with vaginal secretion, so its source of RNA can be the same as blood and vaginal secretion.

1.3.4 Illegitimate transcription of mRNA

It has been demonstrated that a low level of transcription of tissue-specific genes can be detected in a variety of cells [172, 173], for example, β-globin, factors VIIIc and IX, anti-Müllerian hormone, L-pyruvate kinase, retinal blue pigment, and phenylalanine hydroxylase. Illegitimate transcription of tissue-specific mRNA can be used in the analysis of inherited diseases and benefit the diagnosis of these diseases

in biomedical applications [174, 175]. As a consequence, when we try to identify body fluids by detecting their own specific mRNA, false conclusion may occur because of either illegitimate transcription or release of mRNA from distant tissues. Moreover, the level of RNA expression may vary in different individuals as well as tissues, and it can even change over time due to physiological or psychological factors. It is almost impossible to get one specific mRNA which is only expressed in one specific body fluid in all individuals. To deal with this, Roeder *et al.* suggested a minimum of five markers for each body fluid to prevent from false conclusion. A scoring method was also applied to give numerical weight for accurate identification of each body fluid [146].

1.3.5 The applications of RNA analysis to forensic science

Although RNA is less stable than DNA, there are several advantages in using RNA analysis as a tool in forensic science. First, only a small amount of sample is required for RNA analysis. This is an important advantage for criminal investigation because the amount of evidence available may be limited. Another advantage is that DNA and RNA isolation can be conducted simultaneously without more loss of sample [176]. Besides, the expression of mRNA can be cell-specific and stage-specific, which can be used for identifying the type or the physiological condition of biological materials. Thus, it is worthy of applying RNA analysis to forensic science. Moreover, RNA is easily degraded by the ubiquitous presence of ribonuclease enzymes and recent researches have shown that different types of RNA decay at different rates [177]. The relative degradation rate of RNA can be applied to determine the time since deposition for biological materials [7, 178]. The following are applications of RNA analysis to forensic science.

1.3.5.1 Identification of body fluids by using RNA analysis

Although DNA analysis can confirm the presence of biological materials, it cannot determine the type and origin of the evidence in terms of the type of cells or body fluids, which information may be beneficial to criminal investigation [3, 4]. For example, a woman declared that she was sexually assaulted and beaten by the suspect, resulting in bloodstains left at the crime scene. On the contrary, the suspect claimed that he had consensual sex with the woman who was menstruating, resulting in the menstrual blood left on site. In this case, identifying the type of the blood sample is crucial to the forensic investigation. Besides, all of the traditional presumptive or confirmatory methods that have been used for forensic investigation so far are based on microscopic, immunological, chemical, or enzymatic assays. Most of them are time-consuming and require a lot of samples. In addition, some of them are insensitive and some may easily yield false positives. Recently, body fluid identification by analysis of DNA methylation [179-182] and body fluid-specific microbial DNA [88, 181] have been developed as indicators of the presence of specific body fluids in forensic community. Besides, RNA profiling has emerged as a new promising method for the identification of body fluids [144, 146, 147, 183]. Comparing with the present identification methods of body fluids commonly used in forensic science, methods with RNA analysis have equal or higher sensitivity [3, 23, 26, 33, 128].

1.3.5.2 Determination of the age of biological evidence

Several studies have been performed to evaluate the age (the time since deposition) of biological stains, most of which focus on bloodstains. These methods

mainly evaluate changes of the haemoglobin spectral profiles, enzymatic activity, or degradation of RNA. The technologies include high-performance liquid chromatography [184], atomic force microscopy [185], UV-Visible spectroscopy [186], nanophotometer [187], electron paramagnetic resonance spectroscopy [188] and biocatalytic assay [189]. None of these methods have been widely accepted due to poor sensitivity or inadequacy for other body fluids.

RNA is degraded by ribonucleases which are already present in the cell and/or originating from other environmental sources. Different physical and chemical factors may strengthen this effect. The degradation of RNA can be used as an indicator for evaluating the age of biological stains. RNA analysis has been evaluated as a novel tool for the identification of body fluids in forensic science [187, 190]. The degradation rate of RNA also has been reported by Anderson *et al.* for its potential in determining the time since deposition of biological stains [177, 191]. Besides, since RNA degradation can be applied to date a biological stain, it also can be useful for dating other tissues [125]. For example, Hampson *et al.* represented an RNA expression method for aging forensic hair samples [192], and Ohshima proposed that interleukin (IL)-1a, IL-1 b, IL-6, IL-10 and tumour necrosis factor-α are possibly useful markers for wound age determination [193]. In this study, the time since the hair was plucked was estimated by comparison of different RNAs. More introduction and the result are addressed in Chapter 5.

1.3.5.3 Other applications to forensic science

Besides identification of body fluids and determination of the age of biological evidence, RNA analysis can be used for other forensic applications as well. Messenger

RNA pattern can be an indicator of the functional status of cells. For example, vascular endothelial growth factor and hypoxia-inducible factor 1 mRNA transcripts are regulated by oxygen [194] and can be used in post-mortem samples to evaluate the pathological mechanisms leading to death. Smolina *et al.* studied the expression of mRNA for inducible NO synthase in human brain to develop a new approach of post-mortem examination [195]. Besides, Zhao *et al.* also applied quantitative analysis of mRNA transcripts to post-mortem materials and showed significance of investigating degradation profiles prior to carrying out relative quantification of target mRNAs in autopsy materials [196].

1.3.6 Technologies for mRNA analysis

To analyse mRNA markers, RNA is firstly isolated from the biological samples and then the quality and quantity of RNA are evaluated after the extraction [197]. Several strategies for detecting mRNA have been developed in molecular biology, such as northern analysis, nuclease protection assays, in situ hybridization, and RT-PCR. Among these methods, RT-PCR has revolutionized the study of forensic biology. It is used to detect the RNA transcripts of genes. In RT-PCR, an RNA template is transcribed into complementary DNA (cDNA) before further procedures. After PCR, the cDNA is amplified exponentially and can be detected and quantified.

RT-PCR is sensitive in mRNA detection. However, it takes more steps and time for the whole process, which causes more loss and degradation of RNA and a greater chance of contamination. Several methods for amplification of DNA have been revealed beside the PCR technology. Among them, Loop-mediated isothermal amplification (LAMP), developed by Tsugunori Notomi in 2000 [198], is a one-step

DNA amplification with high sensitivity and specificity. More importantly, it is convenient and time efficient. The reaction proceeds at a constant temperature, which means that it can be carried out with minimal and inexpensive equipment at crime scenes. In addition, LAMP can amplify a small amount of DNA within a short time. Hence, comparing with traditional PCR, LAMP is easier, more convenient and efficient. So far LAMP has been used in several fields. In this study, LAMP is the main method used for the identification of blood and saliva. More introduction to LAMP is addressed in Chapter 2 and Chapter 3.

1.4 Aims of this study

RNA has been proven to offer insight into forensic science [7]. This investigation aims to explore several possible uses of RNAs in forensic investigation, including body fluid identification using real-time reverse transcription loop-mediated isothermal amplification (real-time RT-LAMP) and evaluation of the time since a hair was plucked.

Most forensic laboratories use enzymatic tests to identify body fluids before extracting DNA. However, these colorimetric tests often lack both the specificity and the sensitivity required. Hence, identifying the body fluid from which a forensic specimen was originated is still one of the principal challenges in forensic science.

Recently, RNA has been proven to be a better alternative for the identification of different biological materials [136]. With adequate technique it would be possible to identify RNA markers that are body fluid-specific, thereby overcoming the problems of cross reaction. Another advantage of RNA profiling is the requirement of a small amount of starting material, which could be very useful on tiny stains. Thus, the

application of RNA profiling to body fluid identification is worthy of research and endeavour, which is also the motivation of this study.

However, it is required to run the PCR, capillary electrophoresis, or real-time PCR for RNA profiling and these procedures may take a lot of time, which is not suitable for forensic investigation. Thus, seeking a better method for body fluid identification is one of the targets in this thesis. Reverse transcription-loop-mediated isothermal amplification was tested for its capability to identify blood and saliva. In the application of LAMP to RNA profiling, it is required to reverse transcribe RNA into cDNA before LAMP reaction. These two steps (reverse transcription and LAMP reaction) are performed separately and manually. Now, with the technology of RT-LAMP, these two steps are combined into one step of successive procedures as the reagents contain the components for both reactions. Hence, RNA reverse transcription and LAMP are carried out simultaneously.

In this study, a preliminary practice of two-step LAMP (RNA reverse transcription and LAMP reaction are carried out separately) was applied to blood identification in order to evaluate the capability of LAMP application to RNA profiling. Later, RT-LAMP was adopted for body fluid identification. In addition to the target body fluids (blood and saliva), several other types of body fluids (including semen, menstrual blood, vaginal secretion, sweat, and urine) were also tested to confirm the specificity of the markers used for blood and saliva identification. To sum up, the application of RT-LAMP to body fluids identification in this study included the following issues:

- To evaluate the identification of blood with two-step LAMP (including reverse transcription of RNA plus LAMP)

- To evaluate the identification of blood and saliva with by RT-LAMP, including sensitivity, reproducibility, and cross reactivity
- To compare different methods for detection of LAMP products, including electrophoresis, turbidity, and fluorescence detection
- To establish the process flow for identifying body fluids by RT-LAMP

Furthermore, hair samples are usually easily ignored at the crime scene not only because they are too small to be discovered but also it is more difficult to analyse them. As mentioned above, several studies have attempted to determine the age of biological evidence, such as blood. In this study we would like to explore the possibility of determining the time since a hair was plucked at the crime scene. Based on the assumption that different RNAs degrade in different rates, the expression of *18S* rRNA and *ß-actin* mRNA along with the time since the hair was plucked was examined. The aims of dating hair samples are as following:

- To evaluate the possibility of estimating the time since the hair was plucked and its limitation
- To analyse the correlation between the dCq value and the time since the hair was plucked and establish a regression equation by a linear or quadratic regression
- To explore possible impact factors for age determination of the plucked hair

Chapter 2 Materials and methods

This chapter describes methods used throughout the entire course of the research. The detailed experimental designs carried out for specific investigations will be described sequentially in each chapter.

2.1 RNA and cDNA preparation

In this project, total RNA was used as a source of material to identify different body fluids with different methods. Total RNA was first isolated from different body fluids and quantified. It was then reverse transcribed into cDNA before further procedures. Before the RNA extraction, the liquid biological samples should be kept in a refrigerator which maintains a temperature of 2˚C to 8˚C and away from direct sunlight. For the long-term storage, the samples should be stored at -20˚C to halt the degradation process [199]. Preservation reagents could be used to stabilize the samples temporarily at room temperature [200, 201]. If the liquid biological samples are not correctly preserved, the biological material might be destroyed by bacterial growth or inherent degradation [202].

2.1.1 Total RNA extraction

There are several methods for RNA extraction. The basic principle is to break the cells using denaturants and then extract the RNA with organic solvents, like chloroform. The extracted RNA is then precipitated, cleaned, dried and dissolved again. Some commercial kits are available for RNA extraction with good quality. To avoid the

degradation of RNA by RNases, the progress of RNA extraction should be conducted more carefully [128, 203]. It requires a separate working area and a hood dedicated specifically for RNA extraction, along with separate pipettes, RNase-free plastic ware, and regular changing of gloves.

For liquid and dried body fluids tested in this study, total RNA extraction was carried out with RNeasy® Mini Kit (Qiagen® Ltd, UK) according to the protocol for purification of total RNA from animal tissues and with some modifications for different body fluids [204]. The common process is addressed below and detailed descriptions of modifications for some body fluids will be given in Chapter 3.

Buffer RLT (350µl) containing 1% v/v β-mercaptoethanol was mixed with 50µl of liquid sample and homogenized by vortexing for 1 minute. Then, 400µl of 70% ethanol was then added in the solution. The total solution was transferred to the spin column followed by centrifugation at 8000g for 15 seconds. The flow-through was discarded. RNase-free DNase (Qiagen®, Hilden, Germany) was added to prevent the contamination from DNA. Buffer RW1 (700µl) was added to the spin column and then the column was centrifuged at 8000g for 15 seconds. The flow-through was discarded again. The RNA bound to the spin column membrane was washed with 500µl of Buffer RPE twice. The column was placed in a new 2ml collection tube followed by centrifugation at 14000g for 1 minute to eliminate any possible carryover of Buffer RPE. RNase-free water (30µl) was then added to the centre of the spin column, followed by centrifugation at 8000g for 1 minute to elute the total RNA.

For hair samples, RNA was extracted by TRI REAGENT kit (Molecular Research Center Inc., Cat. No: TR118). Except total RNA, DNA can also be extracted

simultaneously by using this kit. Extracted total RNA was kept in DEPC (diethylpyrocarbonate) treated with de-ionized water at -80˚C.

2.1.2 Total RNA quantification

The traditional method for quantifying RNA concentration is performed using a UV spectroscopy. The absorbance of a RNA sample is measured at 260 nm and 280 nm. The RNA concentration can be calculated with the Beer-Lambert Law, which predicts a linear relationship between absorbance and concentration.

In this study, RNA was quantified using NanoDrop® ND-1000 UV spectrophotometer (Thermo Scientific, Wilmington, USA) [205]. A patented sample retention system was used in this method, which allowed the analysis of samples from 0.5µl to 2µl with accuracy and reproducibility.

An amount of 2µl of total RNA was used for the quantification. A blank check was conducted to obtain the reference spectrum in advance. The surface of the measurement pedestal was cleaned with a dry laboratory wipe before the next measurement.

2.1.3 Reverse transcription

Reverse transcription (RT reaction) is a process to synthesise DNA from an RNA template (Figure 2-1). A reverse transcriptase, RNA templates, and a short primer complementary to the 3' end of the RNA are used to direct the synthesis of the first strand complementary DNA (cDNA), which can be used directly as a template for PCR.

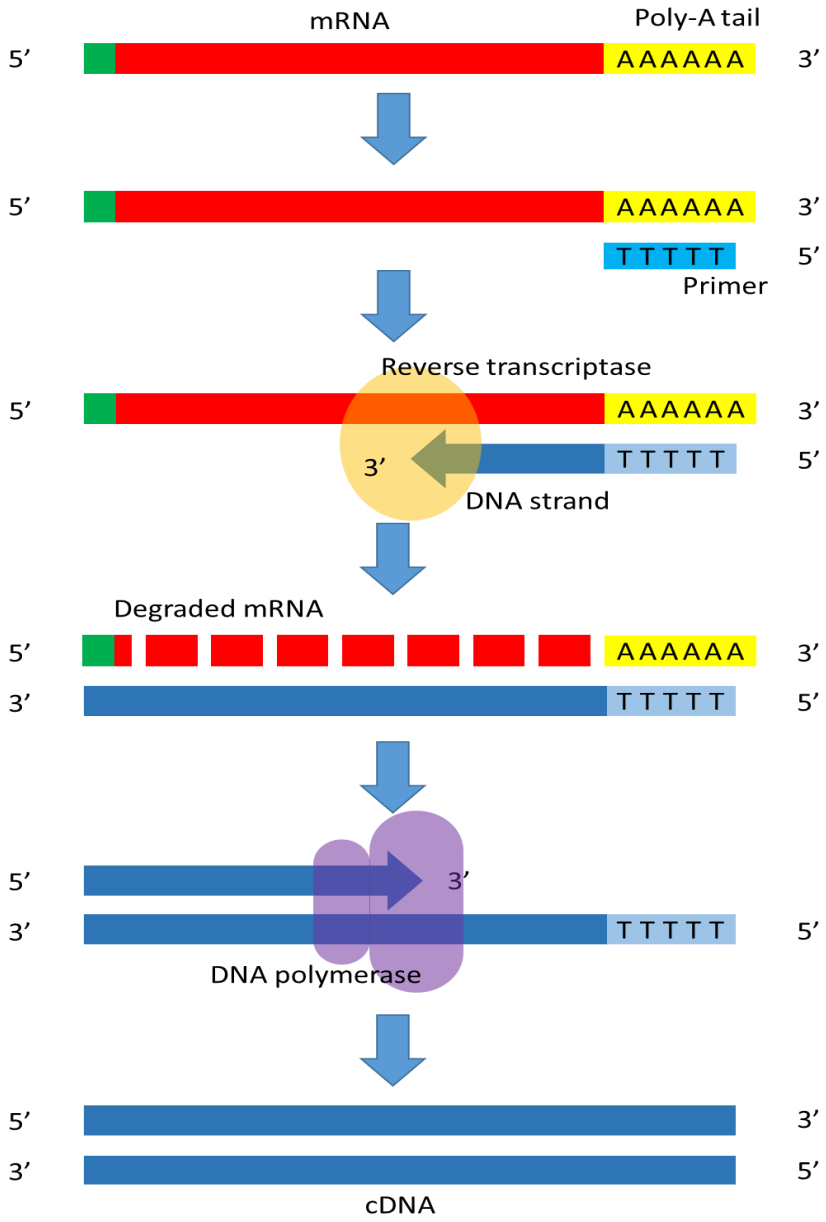


Figure 2-1 Mechanism of reverse transcription and cDNA synthesis

A cDNA is synthesized from mRNA using reverse transcriptase and oligo-dT primers. DNA polymerase uses the mRNA fragments or random primers to synthesize the 2nd strand of the cDNA molecule. Adapters with restriction endonuclease sites or PCR primer sequences can be ligated to the ends of the completed cDNA to facilitate cloning into plasmids or amplification by PCR.

In this study, complementary DNA was synthesized by utilizing a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems®). The RT master mix was prepared as indicated in Table 2-1. The RNA templates were isolated using extractions

with and without RNase inhibitor in order to determine whether or not the presence of the inhibitor would improve the outcome of reverse transcription from the isolated RNA.

Table 2-1 Component and volume for each reaction of reverse transcription

Reagents	1 reaction	Final concentration
10X RT Buffer	2µl	1X
25X dNTP Mix (100mM)	0.8µl	4mM
10X RT Random Primers	2µl	1X
MultiScribe™ Reverse Transcriptase (50U/µl)	1µl	2.5U
RNase Inhibitor	1µl	1U
DEPC Water	3.2µl	
Isolated RNA	10µl	
total	20µl	

RNA (10µl) extracted from each body fluid was added to a 10µl aliquot of master mix. Reverse transcription was performed in a thermal cycler (GeneAmp® PCR System 9700, Applied Biosystems®) using the cycle parameter condition in Table 2-2.

Table 2-2 Time and temperature for reverse transcription reaction

	Step 1	Step 2	Step 3	Step 4
temperature	25°C	37°C	85°C	4°C
time	10 minutes	120 minutes	5 minutes	∞

2.2 DNA amplification and detection

Polymerase chain reaction (PCR) was used for preliminary test in this study. Gradient PCR was used for evaluating the optimization of PCR conditions. The amplified products were then analysed using agarose gel electrophoresis.

2.2.1 Polymerase Chain Reaction

PCR is a molecular biological method to amplify DNA targets *in vitro*. Basically, it requires two specific primers, DNA polymerase, four deoxynucleotides (dNTPs, including dATP, dCTP, dGTP, dTTP) and template DNA [206]. There are three steps for the whole reaction (Figure 2-2). The first step is to denature DNA by heating it to 95°C. After denaturation, the temperature is then reduced to around 60°C, depending on the melting temperature of the primers. Two specific primers are hybridized to the specific sequence of target DNA at this step. At the final step, temperature is raised to 72°C and DNA polymerase extends the primers in 5' to 3' direction by synthesising a complementary DNA in the presence of Mg^{2+} and dNTPs. These three basic steps (denaturation, annealing and extension) make up one cycle of PCR and theoretically double the amount of the target DNA. The newly synthesised strands can then serve as templates, so theoretically there will be 2^n times the target DNA after n cycles. However, the amount of DNA actually does not increase exponentially as theoretically expected due to the consumption of deoxynucleotides and primers and the reducing efficiency of DNA polymerase.

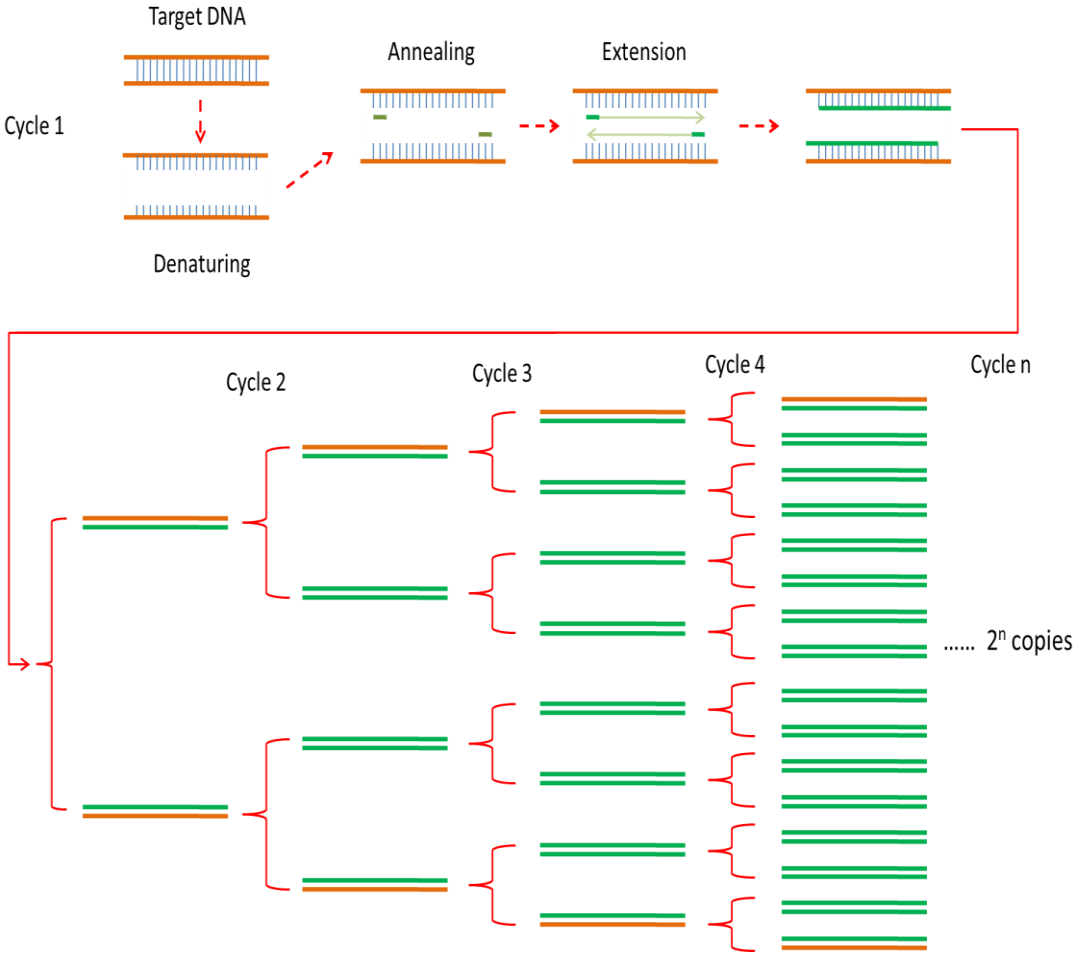


Figure 2-2 DNA amplification by PCR

The temperature of the reaction is controlled to allow 3 steps to take place:
Denaturation (~95°C): The hydrogen bonds that hold the double helix together break apart, separating the DNA molecule into two single strands.
Annealing (~60°C): Primers anneal to the complementary sites of DNA templates. The DNA polymerase binds to the primer and template DNA, and begins the synthesis of the new DNA strand.
Extension (72°C): DNA polymerase adds dNTP's (dATP, dCTP, dGTP, or dGTP) from 5' to 3' to the primer, reading the template from 3' to 5' side, extending from the primer. Bases are added complementarily to the template.
These steps are repeated ~30 times, resulting in an exponential increase in the amount of DNA.

Several key factors will influence the success of PCR. Concerning the chemical components, the purity and integrity of the template (extracted DNA) and primer design are critically important for the reaction. Several software programs are available for designing primers. A successful primer should be designed to be about 18-24 bases long and without internal complementarity to prevent the formation of

secondary structure. The melting temperature (Tm) for a primer needs to be kept around 5-10°C below the annealing temperature. In addition, the proportions of the reaction mixture should be optimized depending on the amount of template.

In this study, complementary DNA obtained from previous reverse transcription was used as a template for PCR. A typical PCR reaction was performed at 1.5mM of $MgCl_2$ as final concentration and at 60°C as annealing temperature. Reaction conditions, e.g. concentration of the $MgCl_2$, annealing temperature, or addition of betaine, were adjusted to improve the amplification. The essential reagents for a typical PCR reaction are listed in Table 2-3.

Table 2-3 A typical PCR reaction

Reagents	per reaction	Final concentration
5XGoTaq buffer (Promega Cat.#M8301)	10 µl	1x
dNTPs, 10mM each (Promega Cat.#C1141)	1 µl	200 µM each
GoTaq polymerase (Promega Cat.#M8301)	0.25 µl	1.25unit
Forward Primer, 10µM	2.5 µl	0.5 µM
Reverse Primer, 10µM	2.5 µl	0.5 µM
Template DNA*	2 µl	50-100ng
$MgCl_2$, 25mM (Promega Cat.#M8301)**	3 µl	1.5 mM
5X Q solution (Qiagen Cat.#203203)	10 µl	1x
H_2O***	X µl	
Total Volume	50 µl	Total Volume

* Complementary DNA from reverse transcription.

** The volume of $MgCl_2$ can be altered for different concentrations.

*** The volume of H_2O can be altered to reach the final volume of 50 µl.

The reactions were performed on a PCR system (Applied Biosystems® GeneAmp® PCR System 9700) using the thermocycling condition, as shown in Figure 2-3.

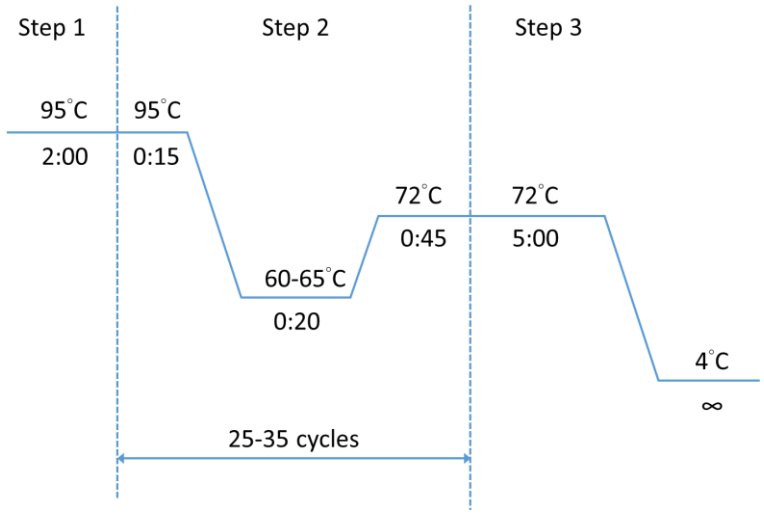


Figure 2-3 A typical PCR thermocycling condition

Thermocycling conditions for a routine PCR usually contain 3 steps. The initial denaturation step prevents DNA from incomplete denaturation. Incomplete denaturation of DNA results in the inefficient utilization of template in the first amplification cycle. After 25-35 PCR cycles, the samples are usually incubated at 72°C for 5-15 minutes to fill-in the protruding ends of newly synthesized PCR products.

2.2.2 Gradient PCR

Although the annealing temperature can be roughly predicted with the theoretical melting temperature (Tm) of the primers, several factors (for example, the sequence and length of primers) may affect the annealing temperature of the thermal cycling reaction for a specific assay. Optimization of PCR conditions is important for improving speed and specificity of PCR. Using a low annealing temperature may produce non-specific products whereas a high annealing temperature may reduce the amount of PCR product. Different annealing temperatures can be set and run in one reaction with a gradient cycler (Figure 2-4), which allows optimization of denaturation, annealing, or extension temperatures in one reaction. In this study, gradient PCR was performed to determine the ideal annealing temperature for a given reaction.

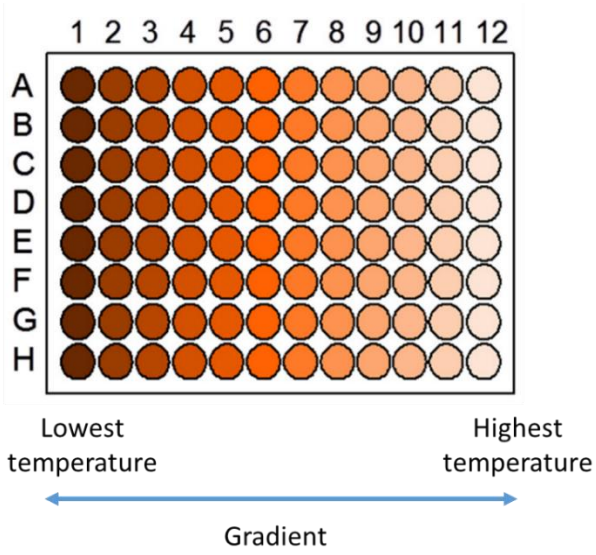


Figure 2-4 An illustration of a 96-well gradient PCR block

2.2.3 Agarose gel electrophoresis

Agarose gel electrophoresis is the most common method to separate, identify or purify DNA fragments. This technique is capable of separating DNA fragments based on their sizes. DNA is a highly negatively charged molecule. As the DNA fragments move towards the positive electrode in the gel, small fragments travel faster than larger ones. Thus, DNA fragments of varying sizes could be separated by electrophoresis. DNA bands of these DNA fragments could be visualized by staining the gel with ethidium bromide (EtBr) or SYBR Green and exposed to UV light. Agarose gel electrophoresis is a simple and cost-effective method for DNA fragment analysis. Also, DNA fragments separated by agarose gel electrophoresis still can be used for following experiments. The length of DNA fragments could be roughly measured by comparing with standard markers. However, the length measurement of DNA fragments by this technique tends to be imprecise (semi-quantitative at best). Thus, further progress with other methods is required for qualitative and quantitative analysis. In addition, the throughput of agarose gel electrophoresis is generally low

[207]. The advantages and disadvantages of agarose gel electrophoresis are summarized in Table 2-4. In spite of the disadvantages, agarose gel electrophoresis still plays a valuable role in scientific research.

Table 2-4 Advantages and disadvantages of agarose gel electrophoresis

Advantages	Disadvantages
● Nontoxic gel medium ● Easy for gel preparation ● Good for separating large DNA molecules ● DNA fragments separated from agarose gel electrophoresis sill can be used for the further analysis	● Fuzzy bands ● Poor separation of low molecular weight samples ● Toxic staining materials ● Low throughput

In this study, a 2% w/v agarose gel was made by adding 8g electrophoresis grade agarose powder (Sigma, Cat. No: A9539) to 400ml 50mM TBE buffer and heated in a microwave oven until the agarose was completely melted. The agarose solution was poured into a gel casting tray with the gel combs and then placed to be solidified at room temperature. TBE stocking buffer (1M) was made prior to the gel electrophoresis. Tris Base (242g) and boric acid (124g) were added and stirred to dissolve in 1L deionized water. Then 14.8g EDTA was added into the solution and dissolved by stirring. The volume of the solution was adjusted to 2L with deionized water. The solution was filtered through a 1 mm filter. The stock solution was then sterilized by autoclaving and stored at room temperature.

DNA samples (3µl) were mixed with 5X loading dye (Table 2-5) and loaded onto the agarose gel and then electrophoresis was conducted at 8 V/cm for approximately 45 minutes to an hour. The gel was then stainned with SYBR® Green I (InvitrogenTM,

Paisley, UK). The PCR product was observed and photographed under UV transillumination (Syngene® GBox, MD, USA) using Genesnap® from Syngene® image acquisition software (Vision-Capt version 14.2).

Table 2-5 Reagents for preparing 5X Loading dye

Reagent	Amount	Final concentration
TBE (1M)	10 ml	250 mM
Sucrose	16 g	40% (w/v)
Xylene cyanol	20 mg	0.05% (w/v)
Bromophenol blue	20mg	0.05% (w/v)
Orange G	20mg	0.05% (w/v)

The volume was made up to 40 ml with deionized water.

2.3 Real-time PCR

2.3.1 Introduction

Traditional PCR is used to amplify the specific target DNA sequence and may be analysed in the endpoint phase by agarose gel electrophoresis [208]. The amount of the PCR product in the endpoint phase can be semi-quantified by comparing the intensity of the band with the standard size marker. The efficiency of PCR is affected by many factors including enzyme activity, the concentration of chemical components, primers and the template. The influence of these factors changes from time to time, so the PCR product is not accumulated as an exponential amplification all the time. In fact, it starts as an exponential amplification in the beginning and then reaches a linear phase. Finally, the efficiency of reaction reduces and the reaction enters a plateau phase since the chemical components, such as dNTPs and primers, are exhausted.

In traditional endpoint PCR, detection of the amplified product is performed at the end of the reaction, involving post-PCR analyses such as gel electrophoresis and image analysis. Real-time PCR (RT-PCR), also called quantitative PCR or qPCR, detects the actual amount of a specific target DNA at each cycle in the PCR reaction and allows the sensitive, specific and reproducible quantification of nucleic acids. Basically, there are three major phases (displayed in the amplification curve) for a complete PCR run based on its efficiency of amplification: the exponential phase, the linear phase, and the plateau phase (Figure 2-5) [209]. In the beginning of PCR (usually around the first 15 cycles), the reaction is just beginning when the emitted fluorescence is so weak that it cannot be distinguished from the background. In the exponential phase, the emitted fluorescence reaches a threshold which is significantly higher than the baseline. When the level of fluorescence from accumulating amplicon reaches the threshold, the cycle number is defined as the quantification cycle (Cq). It is previously known as the threshold cycle (Ct) [210]. The Cq value is inversely correlated to the amount of original nucleic acids [211]. Thus, the quantification of nucleic acids can be achieved by monitoring the change of fluorescence in this phase. However, as the reaction proceeds, some of the reagents are being consumed, which makes the reaction start to slow down in the linear phase. The amount of PCR product is no longer being doubled at each cycle. In other words, the reaction efficiency is not 100% any more in this phase. Finally, one or more of the components become limiting in the plateau phase. The intensity of fluorescence does not increase in this phase.

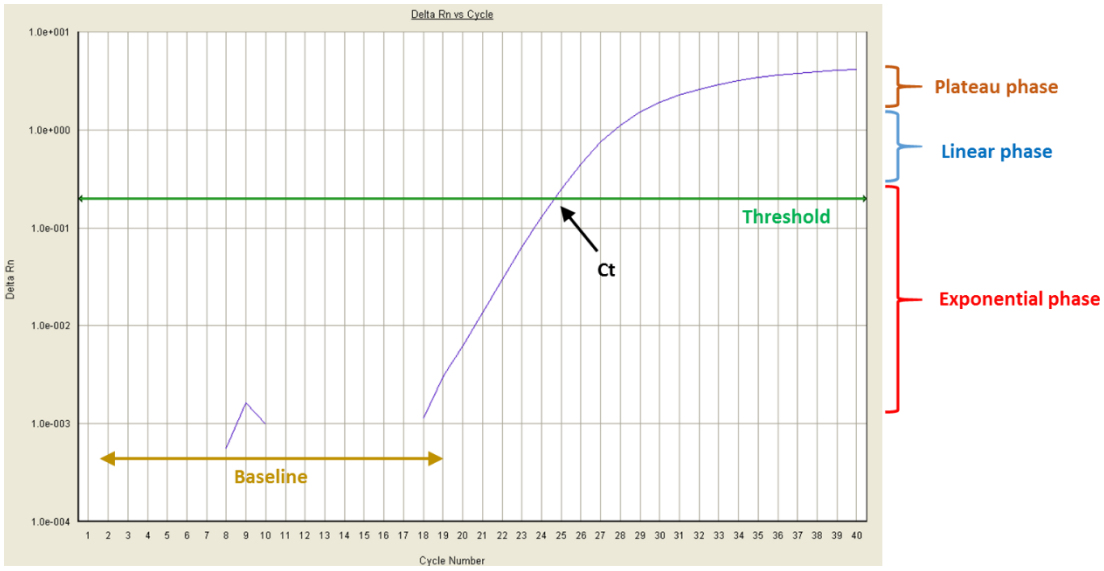


Figure 2-5 A typical amplification plot of real-time PCR

In forensic science, real-time PCR is usually used to quantify the amount of DNA extracted prior to DNA profiling. It is important as the downstream processing can be affected by either too little or too much DNA as starting material. [212-214]. Real-time PCR also has been widely used for the quantification of mRNA, especially in the medical study. Two quantification strategies can be applied in real-time RT-PCR: absolute or relative quantitative real-time RT-PCR.

2.3.2 Methodology

2.3.2.1 Detection of PCR product

In addition to materials for a traditional end-point PCR method, real-time PCR usually requires a fluorescent dye or a fluorescent probe used as an indicator of the PCR product. The intensity of fluorescence increases along with the increase of PCR

product. There are two major fluorescent materials used in real-time PCR for the detection of PCR product: non-specific DNA-binding intercalating dyes and specific fluorescent probes.

(a) Non-specific intercalating dyes

Non-specific fluorescent dyes can be used to intercalate with double-stranded DNA and the amount of PCR product can be measured by the intensity of the emitted fluorescence. SYBR® Green I is one of the most common non-specific intercalating dyes for a real-time PCR [215, 216]. It tends to bind to double-stranded DNA. The complex absorbs the blue light (λmax = 497 nm) and emits the green light (λmax = 520 nm), the fluorescence of which is 1000X stronger than the fluorescence of the SYBR® compound. Though it can also bind to single-stranded DNA and RNA, the performance is relatively lower. So it can still be used as an indicator of double-stranded DNA for real-time PCR. However, as there is no selection for the sequence of target DNA with SYBR® Green I, false positive result might occur due to the intense fluorescence emitted from non-target DNA product.

(b) Specific fluorescent probes

Real-time PCR allows simultaneous amplification and quantification of specific targeted DNA molecules via specific fluorescent probes during the reaction. The fluorescent probe is a short strand of nucleic acid, usually less than 50 nucleotides. Many fluorescent probe-based chemicals have been devised. In this study, TaqMan probes were used to evaluate the PCR product in real time. A fluorescent dye and a quencher dye are attached to each end of a TaqMan probe (Figure 2-6). Sequence-specific DNA probes of oligonucleotides bind to the target gene at the beginning of the reaction. Before the DNA polymerase moves along the template, fluorescence of

the reporter dye is quenched by the quencher dye and hence no fluorescence is detected in this phase. As the polymerase moves on, the probe is cleaved, allowing the reporter dye to detach from the quencher. The unquenched emission of fluorescence can then be detected and calculated for quantification (Figure 2-7).

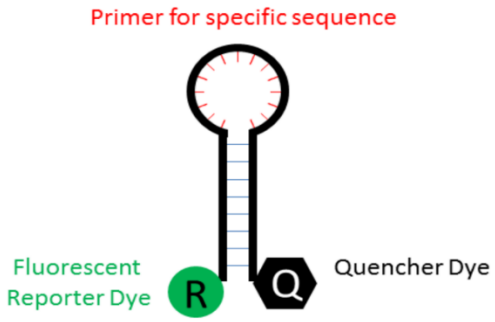


Figure 2-6 An illustration of a fluorescent probe for the real-time PCR

A TaqMan probe is a sequence-specific, fluorescently labeled oligonucleotide. TaqMan assays exploit the 5' to 3' exonuclease activity of certain thermostable polymerases such as *Taq*. The TaqMan probe is labeled with a fluorescent reporter at the 5' end and a quencher at the 3' end. Commonly used fluorescent reporter-quencher pairs are fluorescein (FAM), which emits green fluorescence, and Black Hole Quencher 1 dye.

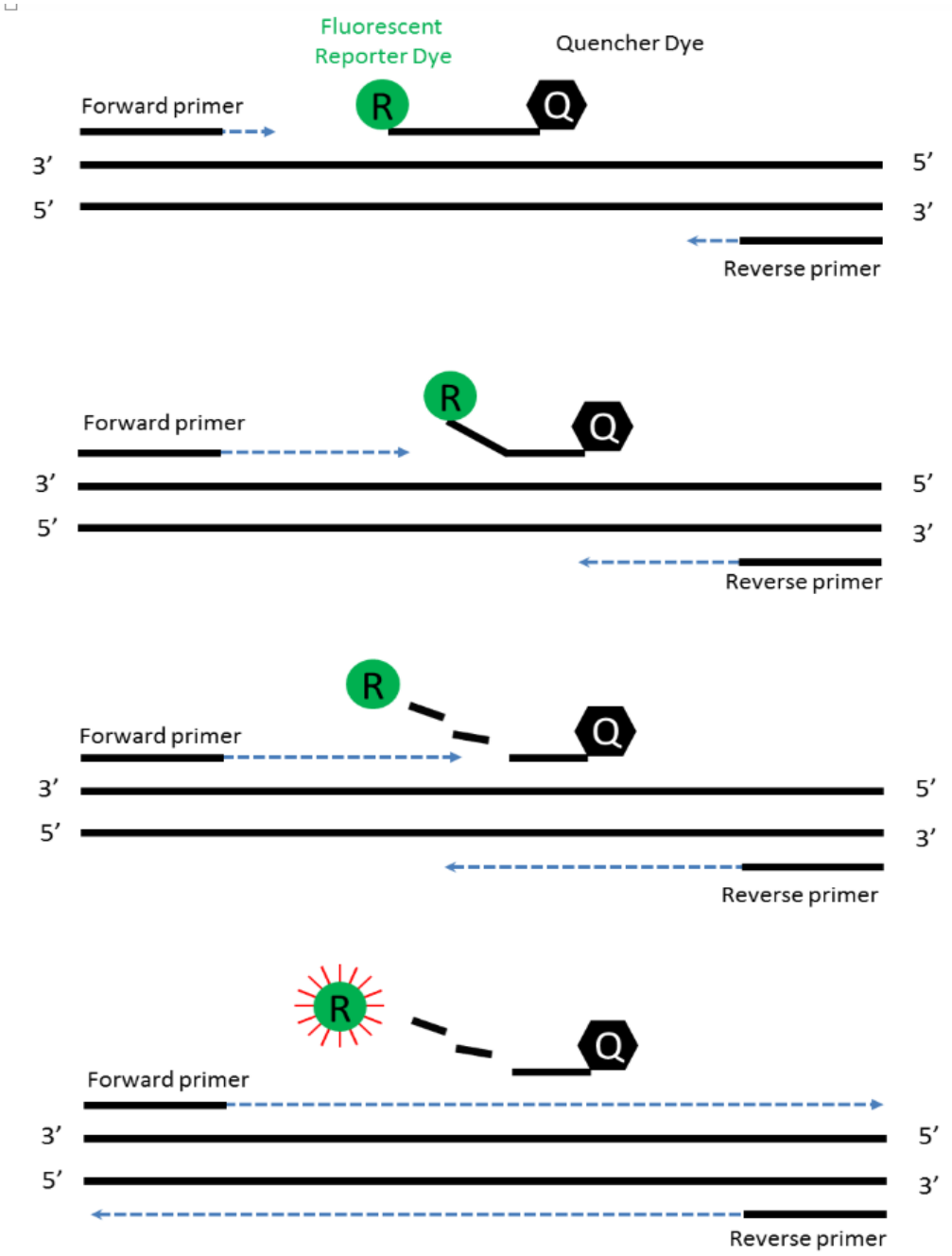


Figure 2-7 An illustration of the real-time PCR with a specific fluorescent probe

When the TaqMan probe is intact, the fluorescence of the reporter is quenched due to its proximity to the quencher. The amplification reaction includes a combined annealing/extension step during which the probe hybridizes to the target, and the dsDNA-specific 5' to 3' exonuclease activity of *Taq* cleaves off the reporter. As a result, the reporter is separated from the quencher, resulting in a fluorescence signal that is proportional to the amount of amplified product in the sample.

The fluorescence is recorded in real time. For a real-time quantification, a threshold for DNA-based fluorescence detection is set slightly above background. The number of cycles in which the fluorescence exceeds the threshold is called the

threshold cycle. Because the concentration of original DNA is inversely proportional to the threshold cycle, the concentration of the temple can be estimated by comparing the standard curve with a series dilution of standard DNA. Most factors which have impact on a PCR can influence the threshold cycle as well, including the effect of master mix components, efficiency of a PCR reaction and dye sets. To evaluate the PCR efficiency, the two factors, value of the slope and r^2, are considered [217]. The value of slope reflects the efficiency of the reaction and r^2 indicates how well one value is predicting another. The quantity can be accurately predicted if r^2 is 1.

2.3.2.2 Absolute quantification and relative quantification

Real-time PCR can be applied to quantify nucleic acids by absolute quantification or relative quantification [218]. Absolute quantification provides the exact result of a target by comparing the result with the calibration curve generated from a series dilution of known template concentration. Therefore, it is required to perform a standard calibration in the same amplification efficiency.

On the other hand, relative quantification is based on an internal reference to determine the fold-differences in the amount of target gene. Relative quantification can also be used to measure the relative change in mRNA expression levels. Relative quantification is easier to perform; it does not require a calibration curve since the quantity of the studied gene is compared directly to the quantity of a control housekeeping gene. In this study, relative quantification was used to evaluate the difference of the decaying rate between different RNAs and more introduction is addressed in Chapter 5.

2.4 Loop-mediated isothermal amplification (LAMP)

2.4.1 Introduction

At present, DNA fragments are usually amplified by PCR reaction to increase the amount of the target template for further analysis. It is required to reversely transcribe RNA into cDNA before the PCR reaction for RNA analysis with this method. Unlike PCR, DNA fragments can be amplified in one step by LAMP since the amplification is based on the strand-displacement activity of the polymerase. LAMP is a one-step DNA amplification reaction with high sensitivity and specificity [219, 220]. The polymerase can amplify the template strand and displace another strand without cleaving it since it lacks the endonuclease activity from 5' to 3'. More details of the LAMP reaction are addressed in Chapter 3.

2.4.1.1 DNA polymerase for LAMP

LAMP reaction employs a DNA polymerase which contains the 5' to 3' polymerase activity, but lacks 5' to 3' exonuclease activity. The DNA polymerase replaces and releases the complementary strands, instead of hydrolysing them (Figure 2-8). Besides, the LAMP reaction takes place at a constant temperature using the DNA polymerase with strand displacement activity, so the previously released strands can be used as a template and amplified with proper primers at once. Via auto-cycling mediated by the DNA polymerase, it keeps displacing target strand DNA and creates new targets as it amplifies.

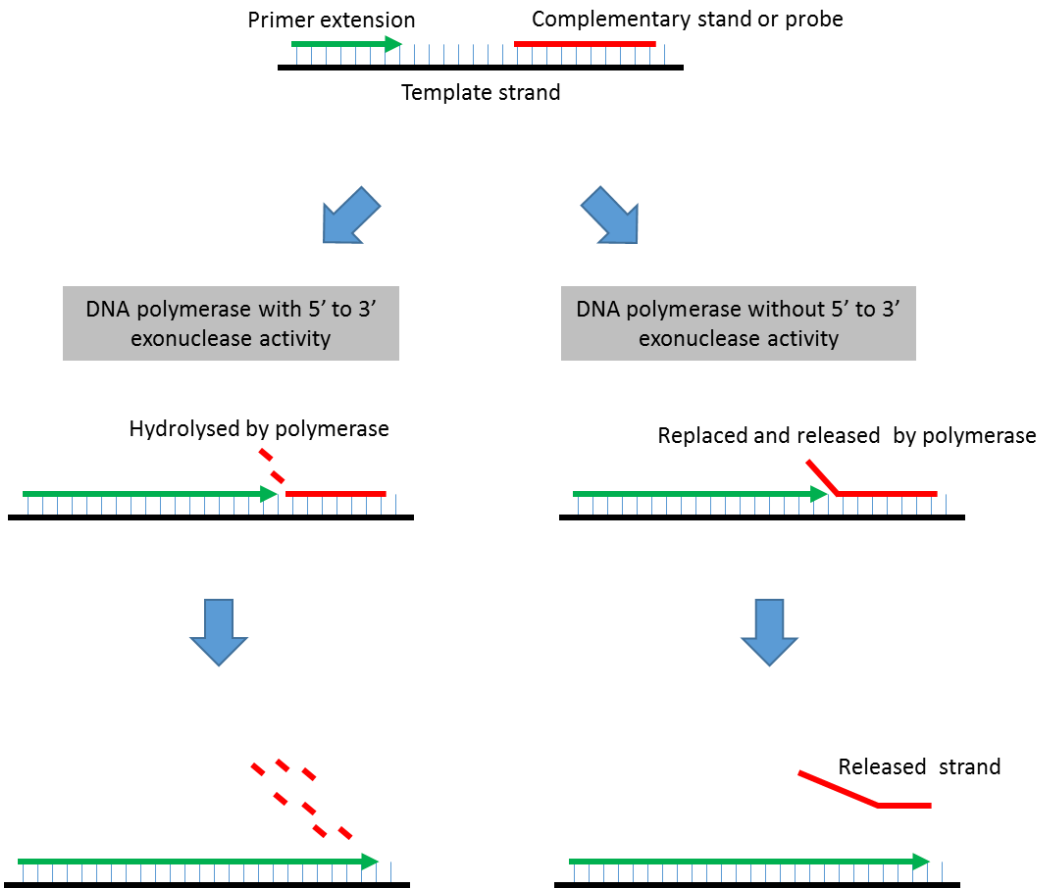


Figure 2-8 Comparison of DNA polymerase with or without 5' to 3' exonuclease activity

Several DNA polymerases, such as *Bst* polymerase, *Bca*BEST DNA polymerase, and Z-*Taq* DNA polymerase, have been used for isothermal amplification. Among these polymerases, *Bst* polymerase is thought to be the best for LAMP amplification [198]. *Bst* polymerase is a DNA polymerase derived from *Bacillus stearothermophilus*. It shows a relatively high enzymatic activity at sub-optimum temperature (Table 2-6) [221]. Besides, it lacks a 3' to 5' exonuclease activity [222]. These aspects make it useful in LAMP reaction. Thus, *Bst* polymerase was used for LAMP reaction in this study.

Table 2-6 Enzymatic activity of *Bst* polymerase at different temperature

Reaction temperature	Enzymatic activity
37°C	10-15%
50°C	30-45%
60-65°C	100%
70°C	20%

2.4.1.2 Primer design of LAMP

The process of LAMP reaction requires at least two primer sets which recognize six specific sequences including F1, F2, F3, B1, B2, and B3 of the target DNA (shown as Figure 2-9 and Table 2-7). The forward inner primer consists of an F2 region at the 3'end and an F1c region at the 5'end. The F1c region is identical to the F1c region of the template sequence. The backward inner primer is made up with a similar manner. Both inner and outer primers hybridize to the complementary sequence of the template DNA in the beginning, resulting in specificity, but only the inner primers are used for the subsequent cycling amplification [223].

Table 2-7 Primer design of LAMP

Primer set	Primer name	Abbreviation	Primer sequence
Inner primers	Forward inner primer	FIP	F2+F1c*
	Backward inner primer	BIP	B2+B1c*
Outer primers	Forward outer primer	F3	F3
	Backward outer primer	B3	B3

* The letter "c" represents the complementary sequence. Thus, F1c represent the complementary sequence of F1 and B1c represent the complementary sequence of B1.

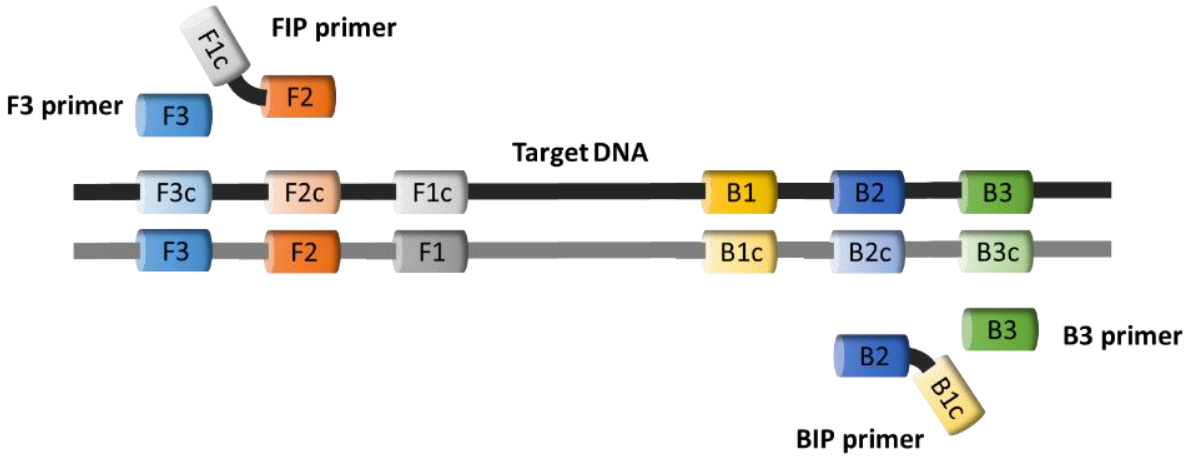


Figure 2-9 Primer design of loop-mediated isothermal amplification

FIP consists of the F2 region (at the 3' end) that is complementary to the F2c region, and the same sequence as the F1c region at the 5' end. BIP consists of the B2 region (at the 3' end) that is complementary to the B2c region, and the same sequence as the B1c region at the 5' end. F3 consists of the F3 region that is complementary to the F3c region. B3 consists of the B3 region that is complementary to the B3c region. Inner primers and outer primers play the role of initiation and self-priming, respectively.

There are 4 key factors in the LAMP primer design with the software. First of all, the melting temperature (Tm) is estimated using Nearest-Neighbour method. The Tm is calculated based on the conditions of experiment such as the concentration of salt and oligo. The Tm is designed between 64-66°C for F1c/B1c and 59-61°C for F2/B2 and F3/B3.

The second factor is the stability at the end of the primers which serves as the starting point of the DNA synthesis. The 3’ end of F2/B2 and F3/B3 and the 5’ end of F1c/B1c are designed to meet the requirement of free energy less than -4 kcal/mol. The change in free energy (∆G) is the difference between the product free energy and the reactant free energy. The annealing between the primer and the target gene is an equilibrium reaction and the annealing reaction proceeds with a smaller free energy.

The next factor is GC content of primers which is better between 40% to 65%. The last factor is the secondary structure. A formation of a second structure may cause the low efficiency of amplification.

One more factor which may affect the reaction is the distance between primers. The primers are designed to amplify a target sequence with a distance ranging from 120 to 160 bases from the end of F2 primer to the end of B2 primer. The primers are also designed to meet the range from 40 to 60 bases from the 5' end of F2 and F1. Finally, the distance between F2 and F3 is between 0 to 60 bases (Figure 2-10). In these regulations, no primers can be designed if the length of the target sequence is too short [224].

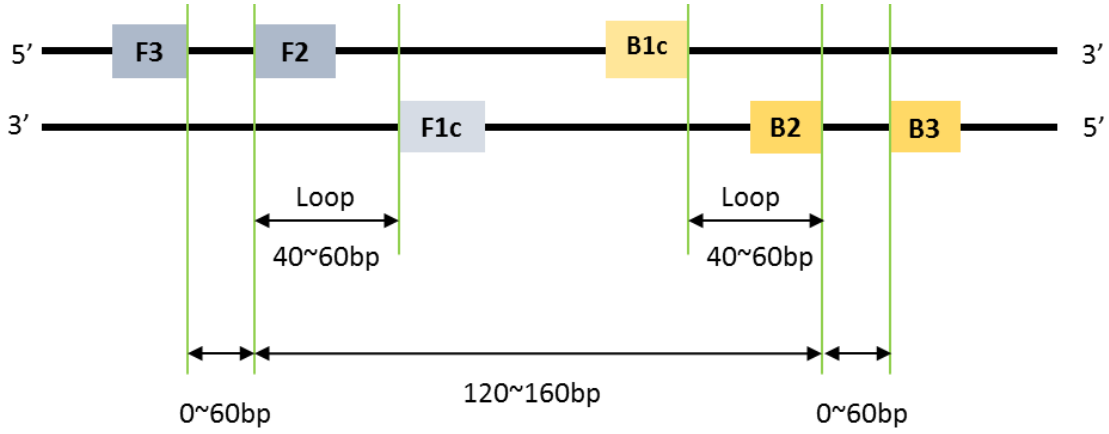


Figure 2-10 Distance between primers

LAMP primers can be designed using the LAMP primer designing software PRIMER EXPLORER V3 (http://primerexplorer.jp/elamp4.0.0/index.html, Eiken Chemical Co., Figure 2-11). After uploading a target sequence file on the website, the

software generates a series of primer sets which can be selected and tested by the user for the LAMP reaction.

Figure 2-11 The website of PrimerExplorer V3 for LAMP primer design

2.4.1.3 Principle of LAMP reaction

There are 2 main steps in the whole LAMP reaction:

- Starting structure producing step
- Cycling amplification step

LAMP amplification initiates from the progress of starting structure producing step which is described as below and illustrated as Figure 2-12 (an animation of the process can be found at http://loopamp.eiken.co.jp/e/lamp/anim.html):

(a) F2 region of FIP hybridizes to F2c region of the target DNA (Figure 2-12 A).

(b) Complementary strand synthesis initiates and the original complementary strand is replaced and released (Figure 2-12 B and C).

(c) F3 primer hybridizes to the F3 region of the target DNA (Figure 2-12 D).

(d) F3 primer extends and displaces the FIP linked complementary strand which is just formed by previous progress. Meanwhile, the FIP linked complementary strand forms a self-hybridizing loop because of the reverse complementary sequence of F1 and F1c at the 5' end (Figure 2-12 E and F).

(e) DNA amplification proceeds with BIP in a similar manner. B2 region of BIP hybridizes to B2c region of the FIP linked complementary strand (Figure 2-12 G) and DNA synthesis initiates again (Figure 2-12 H and I).

(f) B3 primer hybridizes to B3 region of the FIP linked complementary strand (Figure 2-12 J) and synthesizes a new strand, displacing the BIP linked complementary strand (Figure 2-12 K, L and M).

(g) The displaced strand forms a dumbbell-like structure because of the reverse complementary sequence of F1 and F1c at the 3' end, and B1 and B1c at the 5' end (Figure 2-12 N).

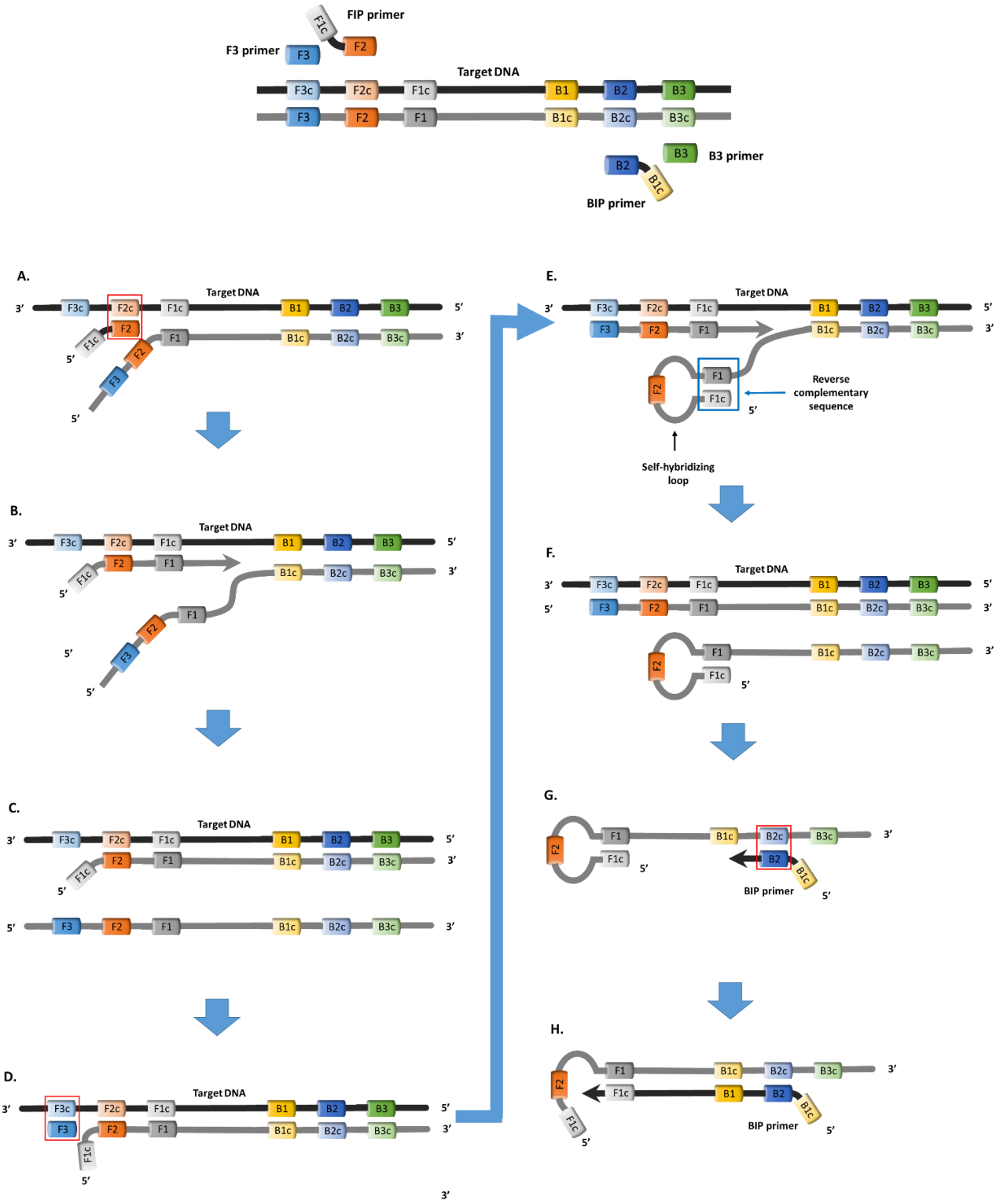


Figure 2-12 The progress of starting structure producing step

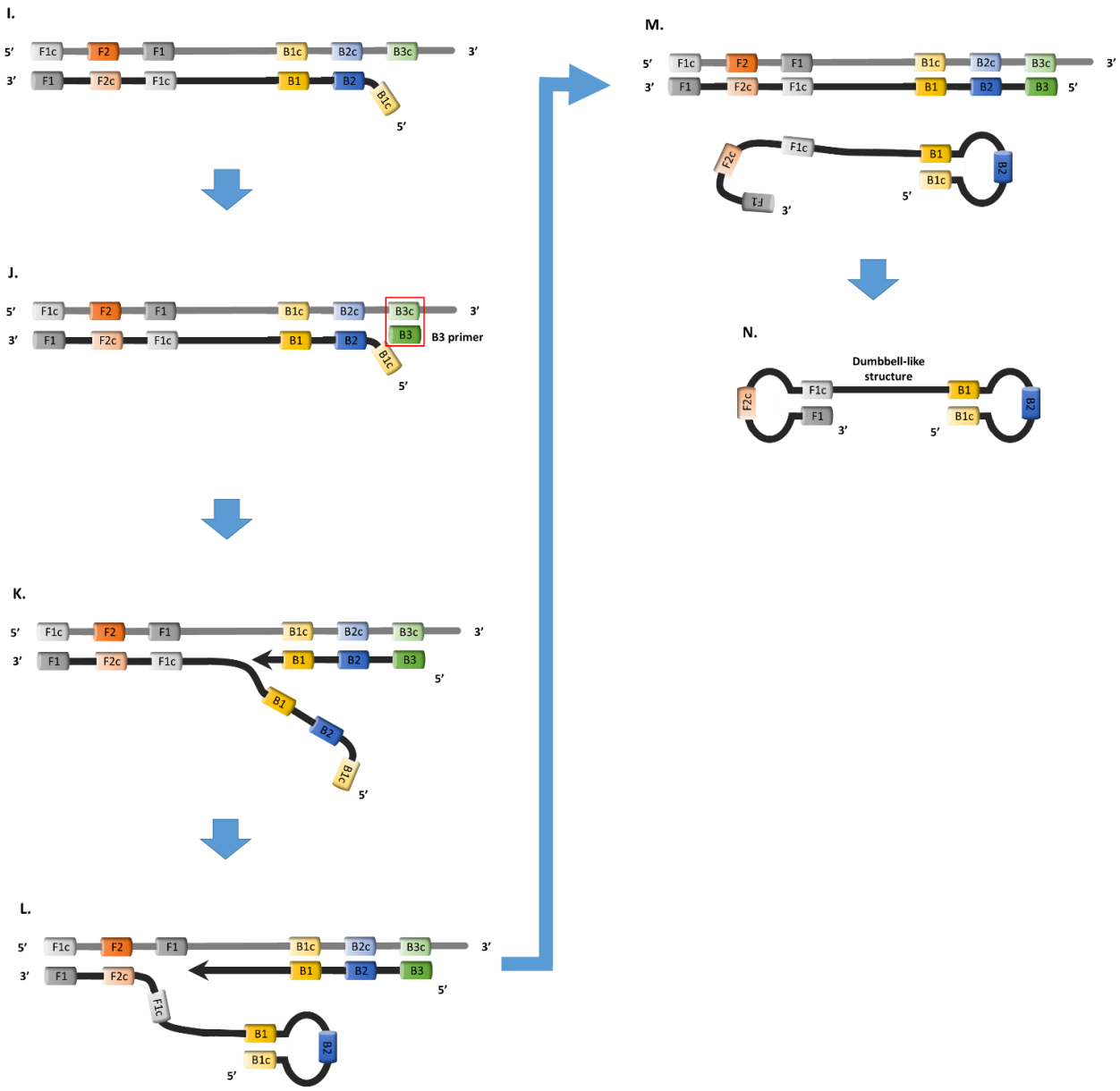


Figure 2-12 The progress of starting structure producing step (continued)

This stem-loop (dumbbell-like structure) synthesized in starting structure producing step serves as a template for a cycling amplification to occur, resulting in strand displacement using the inner primers in the cycling amplification step (cycling amplification step of LAMP, Figure 2-13) [198, 225]. The final product after elongation recycling is a combination of various lengths of amplified DNA (the description given is that of a cauliflower-like DNA structure) [198, 223]. While the reaction might appear

complex, LAMP product can be generated rapidly at a single temperature, thus requiring only one simple piece of heating equipment.

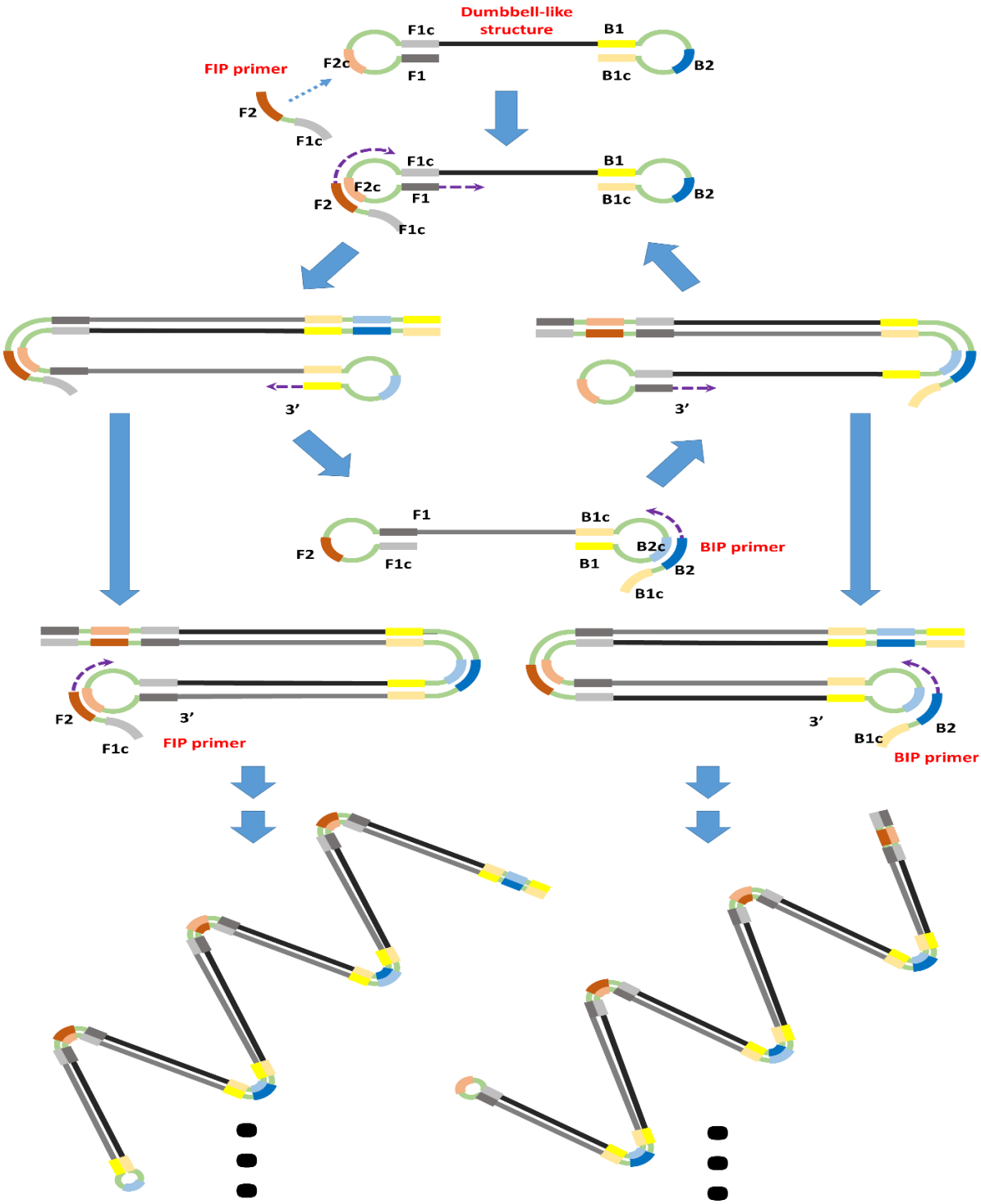


Figure 2-13 Principle of loop-mediated isothermal amplification

2.4.1.4 Advantages of LAMP

LAMP has been widely used in detection of various kinds of pathogens [226, 227], including animals and plants. For example, the virus detection of avian influenza [228] and severe acute respiratory syndrome (SARS) [229]. Besides, the application of LAMP including the identification of microorganism [230], the disease diagnosis of poultry [231, 232] or livestock [233], detection of fungi [234] and parasites [235, 236], study of mycoplasma-like organisms [237]. These pathogens usually spread easily and widely and may cause panic and death. Therefore, a rapid, sensitive, accurate and economical method of diagnosis is very important. Under these considerations, LAMP can be a good candidate for this application. LAMP has also used in the examination of genetically modified organism [238], contamination of food [239], sex determination of embryos [240] and species identification [241]. Moreover, LAMP can be used in detection and prevention of biological terror attacks, such as detection of anthrax spores in spiked soil and talcum powder [242].

Microfluidic electrochemical assay has been developed as an isothermal amplification of DNA in a simple microfluidic chip for detection and quantification of *E. coli* [243]. LAMP technology has also been used for Single Nucleotide Polymorphism Genotyping [244] and the detection of hypermethylated DNA [245]. It is also studied to detect the presence of human DNA in forensic evidence [246].

Denaturing is not required in LAMP reaction. The polymerase separates the two strands of DNA at the same time as amplification. Thus, only one single temperature is required for the whole reaction. Comparing the method with PCR, it is

simple and saves time to perform the reaction since increasing and decreasing the temperature are not required. Moreover, because of the stem-loop structure of the LAMP product, it can be elongated and amplified exponentially and therefore a large amount of amplified LAMP product can be obtained.

Another advantage of LAMP is that LAMP product can be detected simply by conjugating the pyrophosphate produced with magnesium, which forms a white precipitate of magnesium pyrophosphate [247], the accumulation of which can be observed by the naked eye or detected by a turbidimeter in real-time [225]. No more pipetting is required after reagent preparation which reduces the chance of contamination. As an alternative, the fluorescent dye calcein can also be used to detect the LAMP product [248, 249]. The calcein combines with the manganese ion before the reaction. Manganese ion will conjugate with the pyrophosphate ion when the latter is produced during the LAMP reaction and the free calcein combines with the residual magnesium ion. A change in fluorescence can be monitored during the above process. Also, SYBR® Green has been used as a fluorescent detector [250]. The sensitivity of LAMP assay is higher than that of traditional PCR and similar to that of real-time PCR [198, 251].

For RNA analysis, both reverse transcription and LAMP reaction are performed simultaneously in the same tube at the same time (RT-LAMP). The cooperative multitasking reaction not only shortens the required time for the process of body fluid identification but also reduces the chance of contamination because RNA is used directly for LAMP without opening the lid. Harnessing the advantages of real-time RT-LAMP allows the rapid identification of the body fluid type present in a sample.

To sum up, the advantages of LAMP are listed as following:

- Time and labour saving
- Only single temperature is required.
- No energy intensive and precise instrument is required.
- Reduced steps of procedure
- Preventing from contamination
- Economic
- Appropriate for on-site and point of care testing

From the above introduction, LAMP is known as an effective technique for identification of DNA and RNA targets. However, no related application in the forensic field has been developed yet. Hence, in this study, LAMP technique was used as a tool to identify different body fluids. Different fluid-specific RNA markers were chosen and tested as indicators.

In this study, *HBB* was used and evaluated as the specific marker for blood identification and *18S rRNA* as the internal control for RT-LAMP method. The LAMP product was detected with the turbidimeter (named real-time RT-LAMP), agarose gel and/or fluorescence.

2.4.2 Methodology

2.4.2.1 LAMP reaction

In this study, LAMP was carried out with a total 25 µl reaction mixture. Reagents of the reaction mixture are listed in Figure 2-14. The mixture was heated at

95°C for 5 minutes for DNA denaturation, then chilled on ice. Later, 8U *Bst* polymerase large fragment (New England Biolabs) was added, followed by incubation at 60-65°C for 1 hour and heating at 80°C for 10 minutes in sequence to deactivate the reaction. For RNA analysis, RNA needs to be transcribed into cDNA before the LAMP reaction. So it requires two steps for RNA analysis by LAMP reaction.

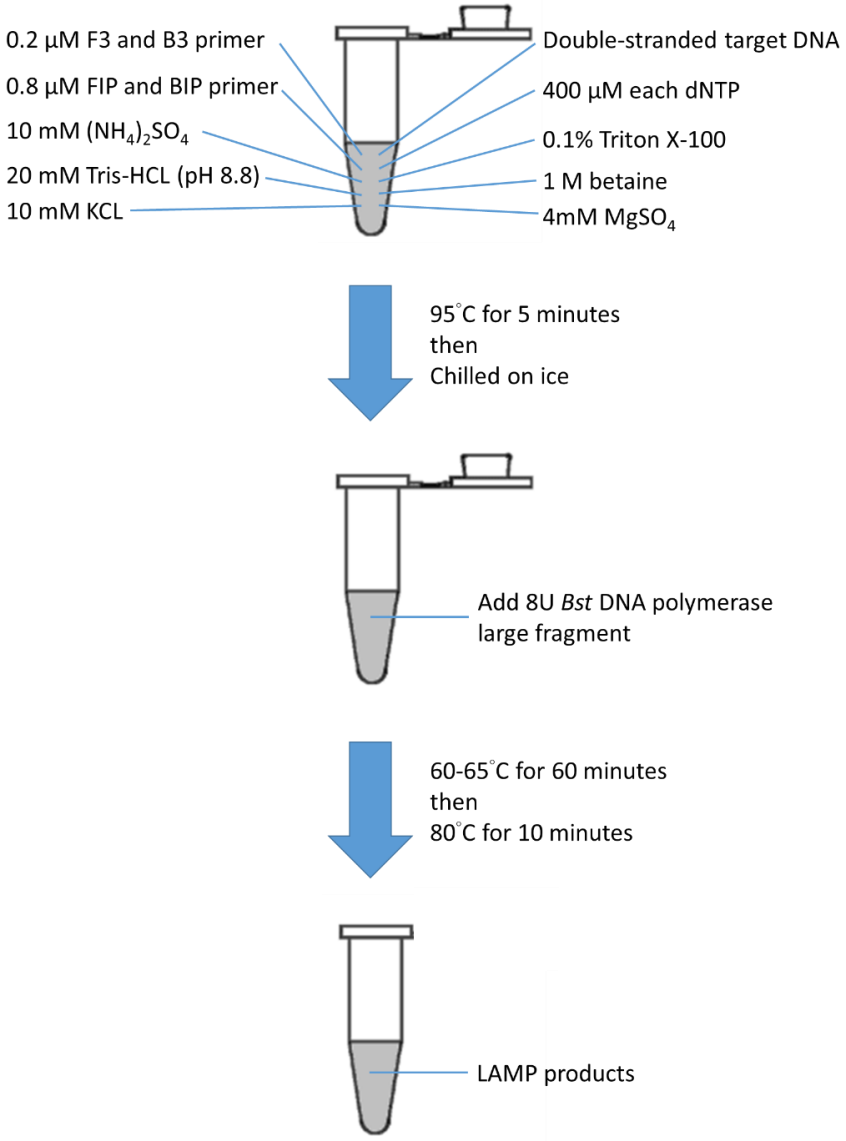


Figure 2-14 A schematic diagram of LAMP reaction

2.4.2.2 Reverse transcription loop-mediated isothermal amplification (RT-LAMP)

It is shown from the above introduction that LAMP can be a potential tool for DNA identification. If it is used for the detection of specific RNA species, cDNA must be obtained from RNA by reverse transcription first. This potentially means more procedures and time, which in turn may make the process prone to loss and degradation of RNA and raise the risk of contamination. To avoid these potential problems, reverse transcription and the LAMP reaction can be performed in the same tube at the same reaction so that the whole process can be done within one tube and in one step [252]. This is the basic mechanism of RT-LAMP.

RT-LAMP provides several advantages for RNA analysis. First of all, it is convenient and cheap in terms of the equipment. Only two temperatures (60-65°C for incubation in the LAMP reaction and 80°C for deactivating the reaction) are enough for the whole reaction as there is no need for denaturation. In fact, it has been shown that LAMP product can be detected even at a single temperature since there is no need to deactivate the polymerase in a real-time detection. In this way, no heavy and expensive thermal cycler is required as one simple heater and one thermometer are sufficient for the reaction to be carried out at a crime scene investigation.

Secondly, it is less labour consuming and less risky. After the sample and reagents are added in the tube, no more pipetting needs to be carried out. The simplification of procedures lowers not only the labour cost but also the risk of contamination and human error.

Moreover, it is effective. It is important to get the result as soon as possible in some emergent cases. A current method of RNA profiling, including reverse transcription and real-time PCR, takes more than 4 hours for the whole analysis.

Comparatively, RT-LAMP takes less than 1 hour to complete the whole reaction. These advantages make RT-LAMP an ideal method for body fluid identification and worthy of a practical application to crime scene investigation.

In this study, a systematic model was established to discriminate blood and saliva from other different body fluids by RT-LAMP. Target RNA was amplified by RNA amplification kit (RT-LAMP, Eiken Chemical Co. Ltd, Japan). Each RT-LAMP reaction was set up as Table 2-8. First, 20µl of master mix was put into each tube, and then 5µl sample RNA was added to the master mix. The total volume for a reaction was 25µl consisting of 40µM for each of the inner primers named FIP and BIP, 5µM for each of the outer primers named F3 and B3 [198], 12.5µl reaction mix which includes 40mM Tris-HCl (pH8.8), 20mM KCl, 16mM $MgSO_4$, 20mM $(NH_4)_2SO_4$, 0.2% Tween20, 1.6M Betaine and 2.8mM for each dNTPs, 1U enzyme (a mixture of *Bst* DNA Polymerase and AMV reverse transcriptase) and approximately 20ng of the total RNA. LAMP was performed using a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) with the thermal program shown in Table 2-9.

Table 2-8 Reagents preparation for a LAMP reaction

Reagents	amount
F3 primer	5 pmol
B3 primer	5 pmol
FIP primer	40 pmol
BIP primer	40 pmol
Enzyme Mix. (EM)	1 µl
2X Reaction Mix. (RM)	12.5 µl
Distilled Water (DW)	X µl
Total	20 µl/test

Table 2-9 The thermal program of a LAMP reaction

	Step 1	Step 2
Temperature	60-65°C	80°C
Time	60-90 minutes	10 minutes

2.4.3 Detection of LAMP product

Several methods can be used for the detection of LAMP product, for example, detection by magnesium pyrophosphate, electrophoresis, fluorescence and real-time turbidimeter.

2.4.3.1 Detection of magnesium pyrophosphate

The LAMP reaction produces pyrophosphate as DNA amplification products are produced. Magnesium ions in the reagent react with pyrophosphate and produce a white precipitate visible to the naked eye [198]. Turbidity can be measured as an indicator for the reaction [247]. However, the observation of precipitate can be subjective and may vary among different observers and sometimes it is not clear whether there is a precipitate or not. For these reasons, more objective methods including electrophoresis, fluorescence observation, and turbidity measured by turbidimeter were adopted to detect LAMP product.

2.4.3.2 Electrophoresis

Because the final LAMP product is a mixture of stem-loop DNA fragments with various lengths and structures (like a cauliflower) with multiple loops, it can be easily confirmed by the electrophoresis [198]. Instead of a single band, a ladder-like pattern can be observed in the agarose gel image of LAMP product after the electrophoresis because of the various lengths and structures of DNA fragments. The significant and clear pattern indicates a positive of LAMP product.

2.4.3.3 Detection by fluorescence

The LAMP product can be detected without opening the lid. The detection is completed via appended fluorescence emitting during the LAMP reaction. Calcein can be used as an indicator of the fluorescent detection [249]. Before the LAMP reaction, calcein binding with manganese ion (contained in the kit) remains quenched. When the LAMP amplification reaction proceeds, the manganese ion is deprived of calcein by pyrophosphate generated by the LAMP reaction. The free calcein tends to bind to magnesium ion in the LAMP mixture which further amplifies the fluorescence [248]. The green fluorescence can be observed under 365nm UV. This principle is applied in the fluorescence detection of LAMP product (Figure 2-15).

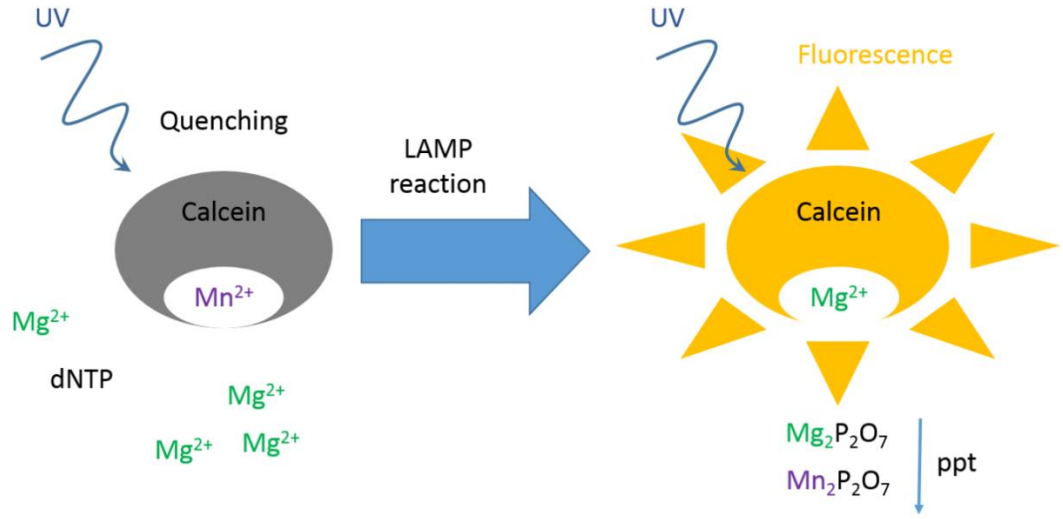


Figure 2-15 The mechanism of detection of LAMP product by calcein

Calcein initially binds with manganese ion (Mn^{2+}) and remains quenched in the beginning. During the LAMP reaction, the manganese ion is deprived of calcein by the generated pyrophosphate, which result in the emission of fluorescence. The free calcein is apt to bind to magnesium ion (Mg2+) in the reaction mixture, which strengthens the fluorescence emission.

Besides calcein, the LAMP product also can be detected by SYBR® Green I (Invitrogen™) which can bind with double-stranded DNA. By adding 0.1U SYBR® Green I to the tube after the LAMP reaction, LAMP product can be observed with green fluorescence under 365nm UV [253]. The LAMP product also can be detected by GelRed™ [231]. However, as this fluorescent dye binds not only with double-stranded DNA but also with single-stranded DNA and RNA, hence it may make false positive result. In this study, LAMP product was detected by fluorescence using calcein and SYBR® Green I as indicators.

(a) Fluorescence detection using calcein

Fluorescence detection was performed using 1 µL calcein (Eiken Chemical Co. Ltd., Tochigi, Japan) added to the RT-LAMP preparations following the

recommendation of the manufacturer (Table 2-10). The working concentration of calcein in the LAMP reaction is approximately 2mM. Fluorescence could be observed by the naked eye using a handheld-UV lamp (wavelength 365 nm).

Table 2-10 Reagents preparation for a LAMP reaction with calcein

Reagents	amount
F3 primer	5 pmol
B3 primer	5 pmol
FIP primer	40 pmol
BIP primer	40 pmol
Enzyme Mix. (EM)	1 µl
Fluorescent Detection Reagent (FD)	1 µl
2X Reaction Mix. (RM)	12.5 µl
Distilled Water (DW)	X µl
Total	20 µl/test

(b) Fluorescence detection with SYBR® Green I

LAMP product was also tested and identified with SYBR® Green I (SYBR® Green I Nucleic Acid Gel Stain, Invitrogen™). 10µl of SYBR® Green I (1/100X dilution) was added to the product after RT-LAMP reaction. The reagent was then observed under 365nm UV with a yellow filtered goggle or under 450-510nm with an aquamarine blue light (Crime-lite® 2, Foster + Freeman Ltd., UK) and an orange filter.

2.4.4 Real-time RT-LAMP

LAMP product can be observed in real-time from the turbidity. A real-time turbidimeter can not only monitor the precipitates, but also determine the optimal cut-off time by a scanning for 1.5 hours in the preliminary test for each marker.

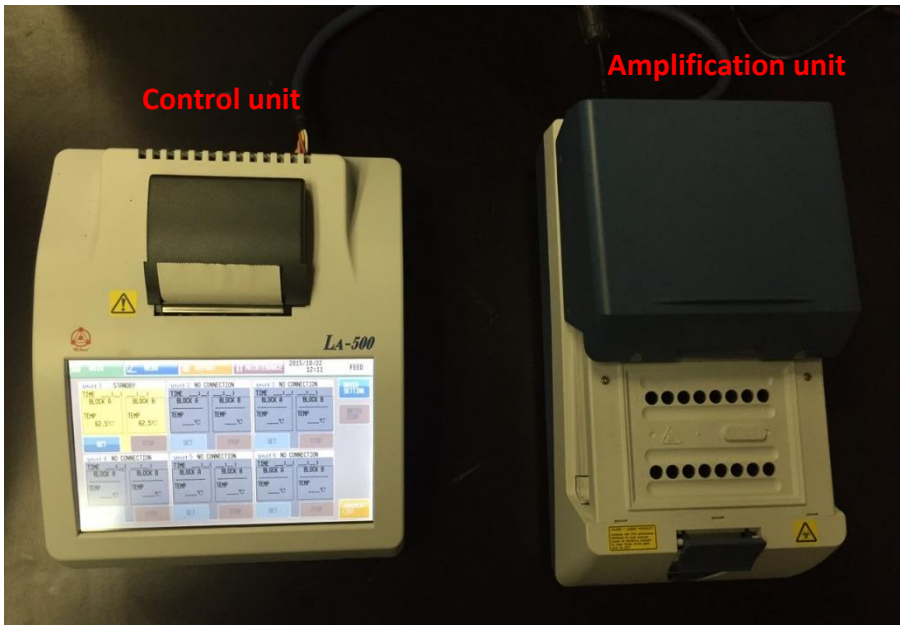


Figure 2-16 Loopamp® Realtime Turbidimeter LA-500

There are two parts in the turbidimeter. The right one is the amplification unit. The left one is the control unit which is a computer and connects with the amplification unit. The amplification unit contains two independent thermal controllers. For each controller, 8 tubes can be detected with the light source (650nm). This device can conduct real-time absorbance detection for 16 samples simultaneously or even more if more amplification units are extended.

In this study, a real-time turbidimeter (Loopamp® Realtime Turbidimeter LA-500, Eiken Chemical Co. Ltd, Japan, Figure 2-16) was used for real-time detection for RT-LAMP. This instrument is a specific device for LAMP reaction, which measures the turbidity at an interval of 6 seconds. The turbidity changes along with the accumulation of the magnesium pyrophosphate in the reaction.

The light (650nm) emitted by light emitting diodes (LEDs) passes through the PCR tubes (containing the LAMP solutions) and is received by the photodiodes (PDs). Both the intensity of LED luminescence (I_{LED}) and the light received by PD (I_{PD}) are recorded (Figure 2-17). As the light will be absorbed by the turbid reagent, resulting in the reduction of the light intensity, the turbidity can be calculated indirectly via counting the absorbance of light intensity based on the following formula [225].

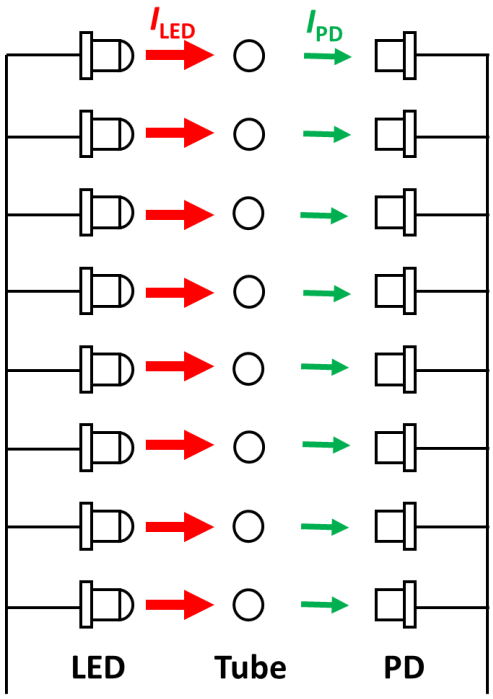


Figure 2-17 An illustration of the real-time turbidimeter used in this study

I_{PD} is the light intensity of light received by PD; I_{LED} is the light intensity emitted by LED. Light (650nm) emitted by 8 LEDs passes through the 8 PCR tubes which opposed to each LED and is received and measured by the PDs. The turbidity can be calculated by the following formula.

$$\text{Turbidity} = \ln (I_{PD} / I_{LED})$$

In this formula, the natural logarithm of the ratio between the light intensity of source and that of the sample is calculated to show the difference of the turbidity

(Figure 2-18). Hence, assuming the final intensity of the light is half of the source, the turbidity absorbance is 0.69 based on the above formula. The turbidimeter measures and calculates the turbidity every 6 seconds and an amplification curve can be made with the data, just like real-time PCR. For example, in Figure 2-19, the stem-loop LAMP product was formed and cycling amplification was carrying on in the beginning. However, the change of the turbidity (ΔAbs) caused by the accumulation of the magnesium pyrophosphate did not exceed the background signal (0-21 minutes). The change of the turbidity was calculated and the threshold time (Tt, min) was recorded as the time when the measurement calculated by moving average differentiation of the turbidity exceeding the threshold according to manufacturer's instructions (Figure 2-20). The study represented by Mori *et al.* [225] indicated that the threshold of LAMP positive for the spectrophotometric measurement was defined as 0.1. The threshold is also suggested and registered on every Loopamp reagent kit by the manufacturer [254]. Tt cannot be obtained if there is no target DNA and no amplification is observed. Errors may be caused by the variation in transparency, the mold of the PCR tubes and variation in insertion condition of the reaction tubes.

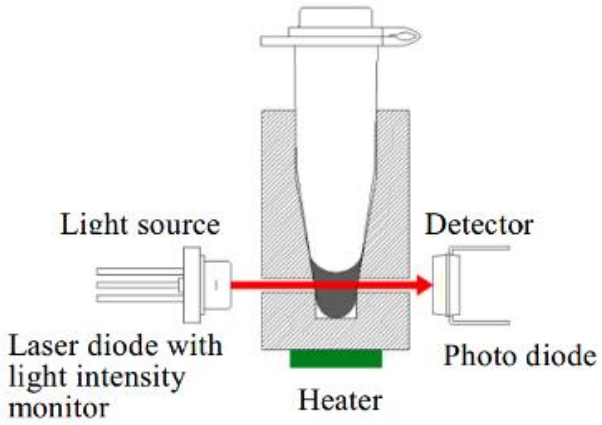


Figure 2-18 An illustration of the detection of turbidity by the real-time turbidimeter

Adopted from Eiken Chemical Co., L., Realtime turbidimeter LA-500 instruction manual. 2010 [254].

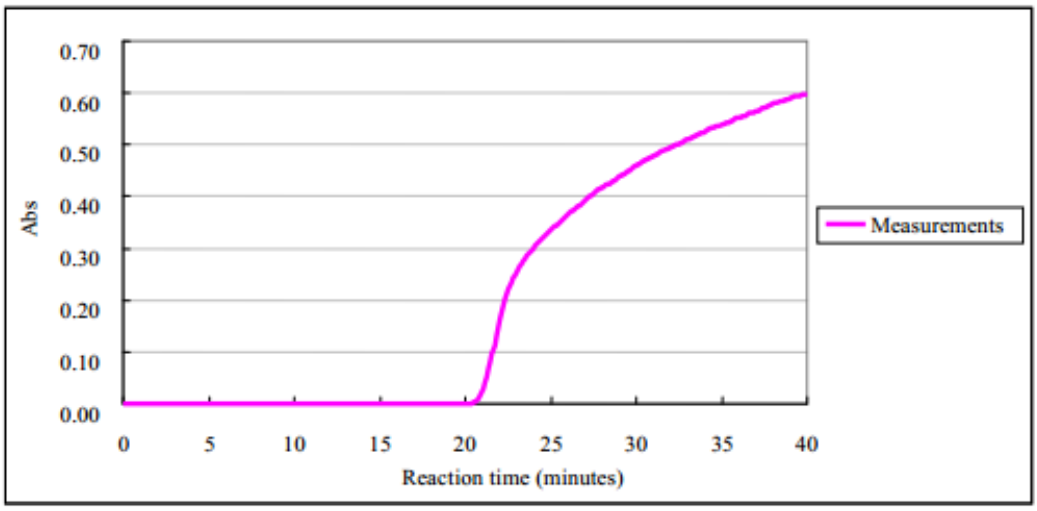


Figure 2-19 Amplification curve for a real-time LAMP

Adopted from Eiken Chemical Co., L., Realtime turbidimeter LA-500 instruction manual. 2010 [254].

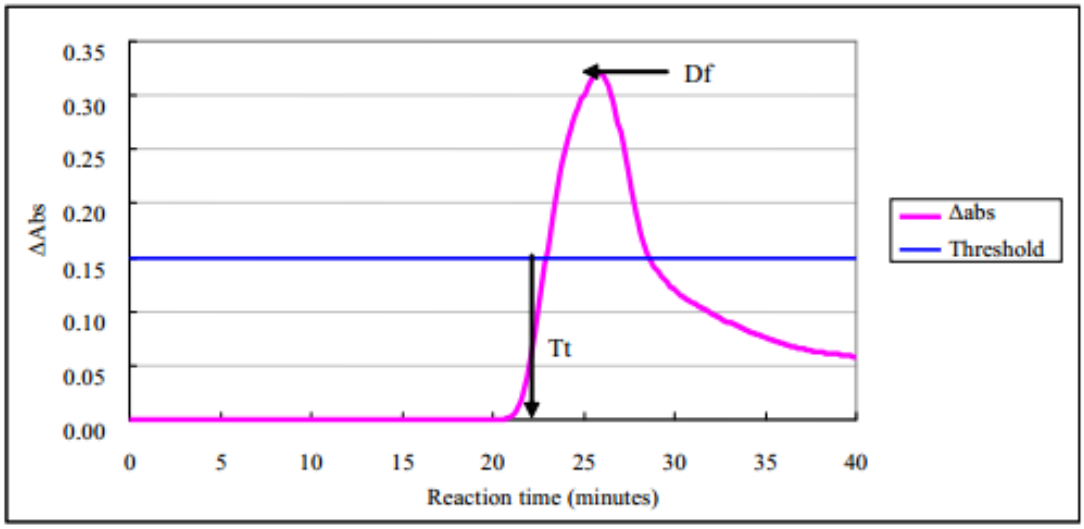


Figure 2-20 The judgment curve for the real-time LAMP

The judgment curve is measured by time-series data of measurements calculated by moving average differentiation. Tt cannot be obtained if there is no target gene and amplification is not observed. Adopted from Eiken Chemical Co., L., Realtime turbidimeter LA-500 instruction manual. 2010 [254].

The reagent preparation and the thermal program for a real-time RT-LAMP reaction are the same as those for RT-LAMP (Table 2-8 and Table 2-9). Instead of using

a PCR thermal cycler, real-time RT-LAMP is performed using the real-time turbidimeter (Loopamp® Realtime Turbidimeter LA-500, Eiken Chemical Co. Ltd, Japan).

2.5 Analysis of the non-probative forensic trace samples

2.5.1 Introduction

To evaluate the potential of real-time RT-LAMP on forensic blood identification, non-probative forensic samples were collected and screened by the presumptive blood tests including Kastle–Meyer test [255] and HemDirect Hemoglobin test (SERATEC®, Göttingen, Germany) [256]. Total RNA extracted from the samples was used for blood identification by real-time RT-LAMP. DNA profiling was also performed to confirm the results.

2.5.2 Kastle-Meyer test

In this project, Kastle-Meyer test, introduced in Section 1.1.1, was used as a presumptive test for the non-probative forensic trace blood samples.

A Kastle-Meyer solution was made by dissolving 2g of phenolphthalein with 100ml of 25% (W/V) potassium hydroxide solution. Twenty grams of zinc powder, which reduced the phenolphthalein into phenolphthalin, was then added to the solution. The reagent consisted of reduced phenolphthalein (phenolphthalin) in alkaline solution (colourless), which was oxidized by peroxide in the presence of haemoglobin in blood. Phenolphthalin (colourless in alkaline solution) was oxidized to

phenolphthalein (intense pink) [12]. The solution was stored in a tightly-capped blue or brown bottle.

2.5.3 Genomic DNA extraction

In this project, genomic DNA was used for DNA profiling to confirm the results of the non-probative forensic trace blood samples in Chapter 3. There are 3 basic steps in DNA extraction. First, cells are broken by cell lysis buffer to expose the DNA within. Then membrane lipids, proteins, and RNA are removed by different ways. Finally, DNA can be purified by different methods, including ethanol precipitation, phenol-chloroform, or mini-column purification.

In this study, genomic DNA was extracted by QIAamp® DNA Mini Kit (Qiagen® Ltd, UK) following the manual's instruction. Briefly, this extraction involved combining 20µl of proteinase K with the blood sample in a 1.5ml tube. Buffer AL (200µl) was added to the sample to lyse the cells. The mixture was incubated at 56°C for 10 minutes. Then 200µl ethanol was added to the sample and mixed by vortexing for 15 seconds. The mixture was applied to a QIAamp spin column (in a 2ml collection tube) and centrifuged at 8000rpm for 1 minute. DNA has a high affinity for binding onto QIAamp silica membrane while contaminants pass through. Two washes were carried out by adding 500µl buffer AW1 and AW2, and centrifugation at 13,000rpm for 1 minute. These two wash steps removed residual of protein and other contaminants which may inhibit PCR. Then a QIAamp spin column containing DNA was placed into a clean 1.5ml tube. 200µl buffer AE was added to the column and incubated at room temperature for 1 minute and spun at 13,000rpm for 1 minute to elute the DNA.

2.5.4 Quantification of DNA

Forensic DNA analysis is targeted to obtain a DNA-STR profile from biological samples. The profiling procedures involve DNA extraction, quantification, amplification, and fragment analysis. Quantification of DNA is required before amplification because the quantity of DNA plays an important role in a PCR reaction. Too little DNA may produce only partial profiles whereas too much DNA may produce off-scale data.

In this project, genomic DNA extracted from the non-probative forensic trace blood samples addressed in Chapter 3 was quantified by real-time PCR assay. DNA was quantified by using 7500 Real-Time PCR System (ABI, Foster City, CA, USA) with Quantifiler™ Human DNA Quantification Kit (ABI, Foster City, CA, USA) which is based on real-time PCR targeting human telomerase reverse transcriptase gene [257] and validated for human identification applications. Each reaction was set up as Table 2-11. Besides, 8 DNA quantification standards (dilution series with the concentrations raging from 50ng/µl to 0.023ng/µl) and 2 reactions for each standard were prepared as Table 2-12 and applied for the standard curve. The concentration of the samples could be evaluated by comparison with the standards.

Table 2-11 Reagents preparation for a human DNA quantification

Reagents	per reaction
PCR Primer Mix	10.5 µl
Reaction Mix	12.5 µl
Genomic DNA	2 µl
Total Volume	50 µl

Table 2-12 Preparation for the standard dilution series with the concentrations raging from 50ng/µl to 0.023ng/ul

Standard No.	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8
H_2O	30µl	20µl	20µl	20µl	20µl	20µl	20µl	20µl
+10µl	Standard	STD1	STD2	STD3	STD4	STD5	STD6	STD7
Concentration (ng/µl)	50	16.7	5.56	1.85	0.62	0.21	0.068	0.023

2.5.5 DNA profiling

DNA profiling is a technique that helps forensic investigators identifying individuals by characteristics in some special regions of the human genome. A DNA profile can be compared with other DNA profiles in a database. If DNA profiles obtained from a suspect and a sample are indistinguishable, it is highly possible that the sample originated from the suspect. Nevertheless, it cannot be excluded that the sample might come from a random person, instead of the suspect, even though the probability is extremely low. The calculation of the rarity of the given DNA profile can help to estimate the match probability. It can be calculated with a proper population data by statistical methods such as random match probability [258].

Several DNA profiling techniques have been reported and used in forensic science, including RFLP, VNTRs, STRs and SNPs. Among these methods, STRs system is used by most of the forensic scientists all over the world. STR markers are polymorphic DNA loci that contain a repeated nucleotide sequence. The STR repeat unit is usually 2-8 nucleotides in length. The length of each allele fragment varies with the repeated numbers contained. In each STR locus, the homozygote or heterozygote can be observed by fragment analysis of the amplified product (for example: electrophoresis) and the exact numbers of repeating units are measured by comparison to an allelic

ladder. The combination of marker sizes makes up the profile of the STR locus. An example is shown in Figure 2-21. When multiple STR loci are examined at the same time, the full profile of the sample could be obtained for comparison purposes. An example of the electropherogram plot of multiple STR loci and its DNA profile are shown in Figure 2-22.

In this project, DNA profiling was performed to confirm the result of the samples from the non-probative forensic trace blood samples in Chapter 3. After genomic DNA was extracted from the evidence, STR loci were amplified using PCR technology. The PCR products were then analysed by electrophoresis to separate the alleles according to size. PCR-amplified STR alleles were detected using fluorescent dye labelling.

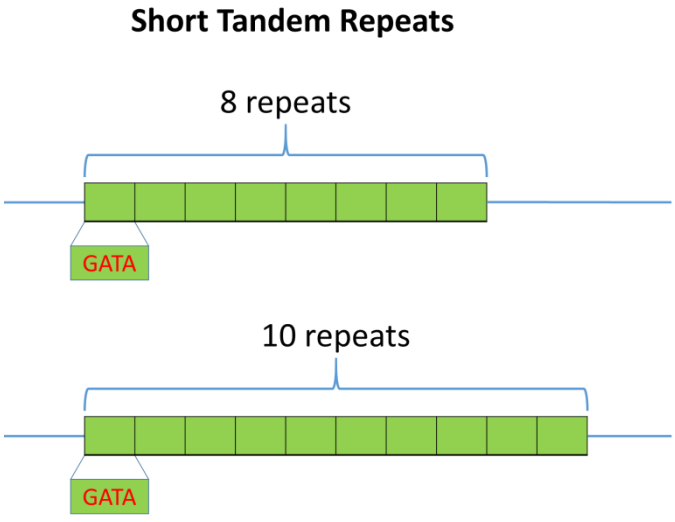
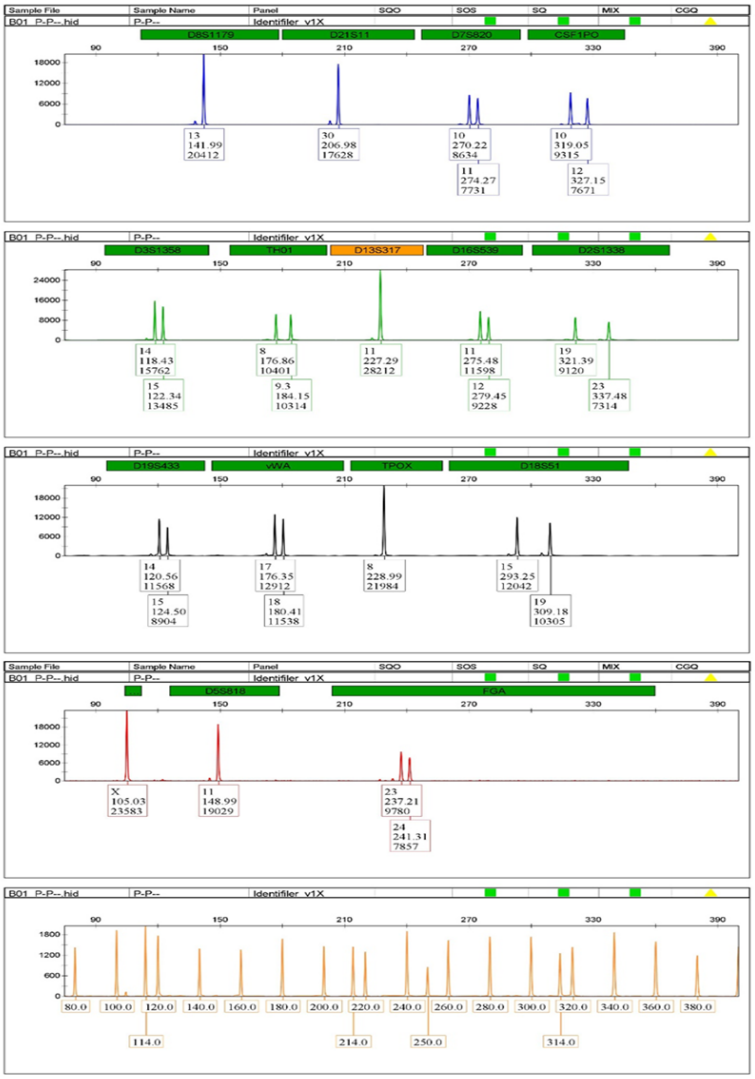


Figure 2-21 Schematic representation of an STR marker (4 base pairs in one repeat unit)

The above shows an example of STR. The top strand represents one copy of one person's DNA. The bottom strand represents the second copy of that person's DNA. The person's DNA has inherited 8 and 10 repeats of the tandem repeat region from his/her parents. Thus, his/her STR profile in this locus can be presented as (8, 10).



STR loci	Alleles
D8S1179	13,13
D21S11	30,30
D7S820	10,11
CSF1PO	10,12
D3S1358	14,15
TH01	8,9.3
D13S317	11,11
D16S539	11,12
D2S1338	19,23
D19S433	14,15
VWA	17,18
TPOX	8,8
D18S51	15,19
Amelogenin	X,X
D5S818	11,11
FGA	23,24

Figure 2-22 An example of a DNA profile

An example of a profile (left) generated from a positive sample using AmpFlSTR® Identifiler® PCR Amplification Kit. The DNA profile of the sample showing the STR alleles is listed in the right.

2.5.5.1 Amplification

The AmpFlSTR® Identifiler® PCR Amplification Kit (ABI, Foster City, CA, USA) was used to co-amplify the repeat regions of the following 16 STR, named D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TOPX, D18S51, Amelogenin, D5S818, and FGA [259]. Among these loci, Amelogenin is located on the X and Y chromosomes at Xp22.1-Xp22.3 and Yp11.2 [260],

which is used in sex determination for unknown human samples. Each PCR amplification was set up as Table 2-13.

Table 2-13 The volume of each component needed to prepare the reactions

Reagents	per reaction
AmpFlSTR® PCR Reaction Mix*	10.5 µl
AmpliTaq Gold® DNA Polymerase**	0.5 µl
AmpFlSTR® Identifiler® Primer Set***	5.5 µl

After the mixture was prepared based on the above table, 15µl of the mixture was dispensed into each reaction well of a MicroAmp® Optical 96-well Reaction Plate. Total DNA (1 ng in a final volume of 10µl) was added to the reagent mixture. The final reaction volume was 25µl.

*AmpFlSTR® PCR Reaction Mix contains MgCl2, deoxynucleotide triphosphates, and bovine serum albumin in buffer with 0.05% sodium azide.

** AmpliTaq Gold® DNA polymerase contains enzyme with an activity of 5 U/µl.

*** AmpFlSTR® Identifiler® Primer Set contains fluorescently labeled primers and non-labeled primers.

One primer of each locus-specific primer pair was labelled with fluorescent dye, including 6-FAM™, VIC®, NED™, and PET®. The fluorescent dyes could be detected in different wavelengths, which allowed them to be detected at the same time. The reactions were run on a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems™, Foster City, CA, USA) with the thermal program shown in Table 2-14.

Table 2-14 Program of the thermal cycling conditions

Step	Initial incubation step	Denature	Anneal	Extend	Final extension	Final hold
Condition	Hold		Cycle (28)		Hold	Hold
Temperature (°C)	95	94	59	72	60	4-25
Time (min)	11	1	1	1	60	∞

2.5.5.2 Capillary electrophoresis

The amplified multiplex STR loci were separated on the genetic analyser (Applied Biosystems, Foster City, CA, USA) which is a capillary electrophoresis system used for analysing fluorescently labelled DNA fragments. Each PCR product (1.5µl) was mixed with 9µl Hi-Di™ Formamide (ABI, P/N: 4311320) and 1µl GeneScan™ 500 LIZ® Size Standard (ABI, P/N 4322682) and then heated at 95°C for 3 minutes. The denatured mixture was snap-cooled on ice for 3 minutes to prevent DNA secondary structures from reannealing [261]. The PCR products were separated in a capillary filled with polymer solution (POP™-4; P/N: 402838). To achieve high precision for sizing DNA fragments by electrophoresis, GeneScan™ 500 LIZ® was used as an internal-lane size standard. GeneScan™ 500 LIZ® is designed for sizing DNA fragments in the 35 - 500bp range, and provides 16 single-stranded fragments of 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, and 500 bases.

The allelic ladder was used like a molecular ruler to help evaluate the lengths of the DNA fragments. It contains common STR alleles that have been previously characterized for the number of repeat units, which is used to calibrate PCR product sizes to STR repeat number for the following genotyping (Figure 2-23).

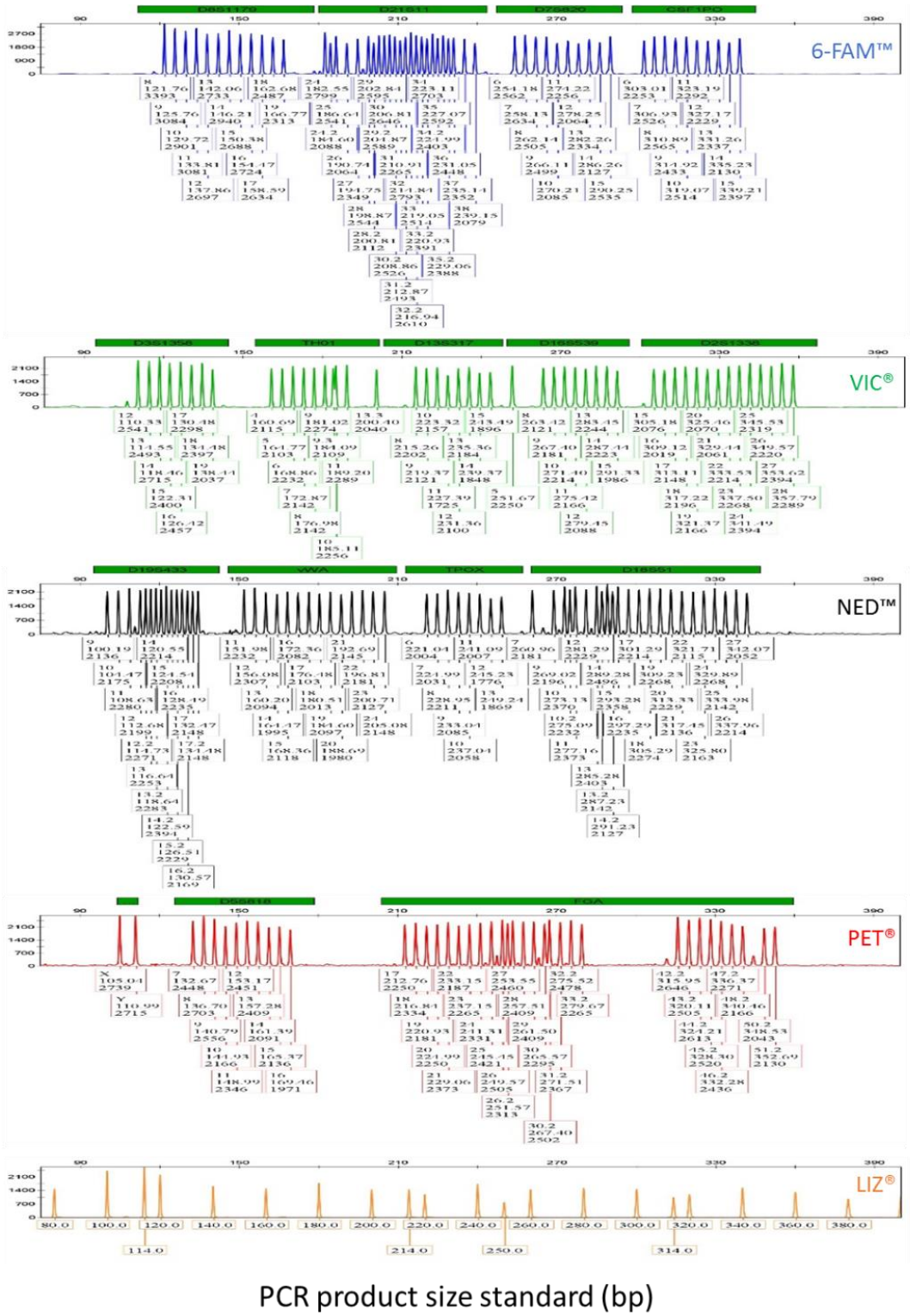


Figure 2-23 An allelic ladder of AmpFlSTR® Identifiler® molecular markers containing 16 STR loci

Allelic ladders contain all the alleles at all loci. Since the allelic ladders and the DNA fragments have the same length, the size of the fragments can be evaluated when conducted with allelic ladders.

To generate a DNA profile, the collected data in the form of multi-coloured electropherograms were then analysed. The results could be displayed separately in each colour (Figure 2-22).

Chapter 3 Identification of blood by loop-mediated isothermal amplification

3.1 Introduction

Blood is encountered frequently during a forensic investigation where it is standard practice to perform STR typing for human identification. The identification of body fluid(s) from which the DNA profile arose is now possible using the mRNA extracted from the sample [3, 7]. Analysis of RNA has been found to be a specific test for a range of body fluids [146], which is sensitive and stable [127, 262, 263]. Recent studies also have shown the potential of microRNA [5] and DNA methylation to identify body fluids through differential patterns [180].

Typically, the RNA is reverse-transcribed into cDNA prior to PCR amplification. The presence or absence of tissue-specific markers is either monitored by the use of real-time PCR [128, 264] or the products detected by capillary electrophoresis [148]. Though these methods have been proved to be very successful in forensic practice, they are still time consuming with multiple steps and with the potential loss of sample consequently [265]. In addition to PCR, there are also other methods to amplify DNA, such as self-sustained sequence replication (3SR) [266], nucleic acid sequence-based amplification (NASBA) [267], strand displacement amplification (SDA) [268], rolling-circle amplification (RCA) [269], ligase chain reaction (LCR) [270] and loop-mediated isothermal amplification (LAMP) [198]. All of the above methods have their own features and advantages for the amplification of DNA.

Another method to be developed for body fluid identification is Reverse Transcription-Loop Mediated Isothermal Amplification (RT-LAMP), in which reverse

transcription and subsequent LAMP reaction can be performed in one tube [271]. The LAMP technique was developed by Notomi *et al.* [198, 223] and has been used widely for rapid detection and identification of parasites and diseases [272-274] and even in determining the sex of embryonic animals [275]. The only two applications of LAMP in a forensic context to-date were detecting bacterial strains relevant to those in saliva [276] and identification of human DNA by LAMP combined with a colorimetric gold nanoparticle hybridization probe [246].

Compared with traditional PCR, LAMP is a simple, rapid, specific and cost-effective nucleic acid amplification method and suitable for application at crime scenes due to the following features. The whole reaction can be carried out only with a normal heater or water bath at 60 to 65°C, so it can be applied to field investigation. Hence, no energy intensive and precise thermal cycler is needed in LAMP reaction since only single temperature is required for the polymerase of LAMP. In addition, reverse transcription and LAMP reaction can be performed simultaneously, which speeds up the reaction process and reduces the chance of contamination. The combination of reverse transcription with the LAMP has great potential for the forensic detection of body fluids. Besides, several studies showed that LAMP reaction is more sensitive than PCR [277-279].

In this study, a proof-of concept model was established first to discriminate blood from other different body fluids by loop mediated isothermal amplification. A specific marker (*HBB*) for blood and a control marker (18S) were selected. The optimal primer sets of RT-LAMP for blood identification were designed and different detection methods were tested. It was hypothesized that LAMP might have superior sensitivity than current methods (such as PCR) and be highly specific to a target locus. The

advantages of using one piece of equipment and speed of reaction were also reasons to apply this method to case samples.

3.2 Methods

The flowchart of this study is shown as Figure 3-1:

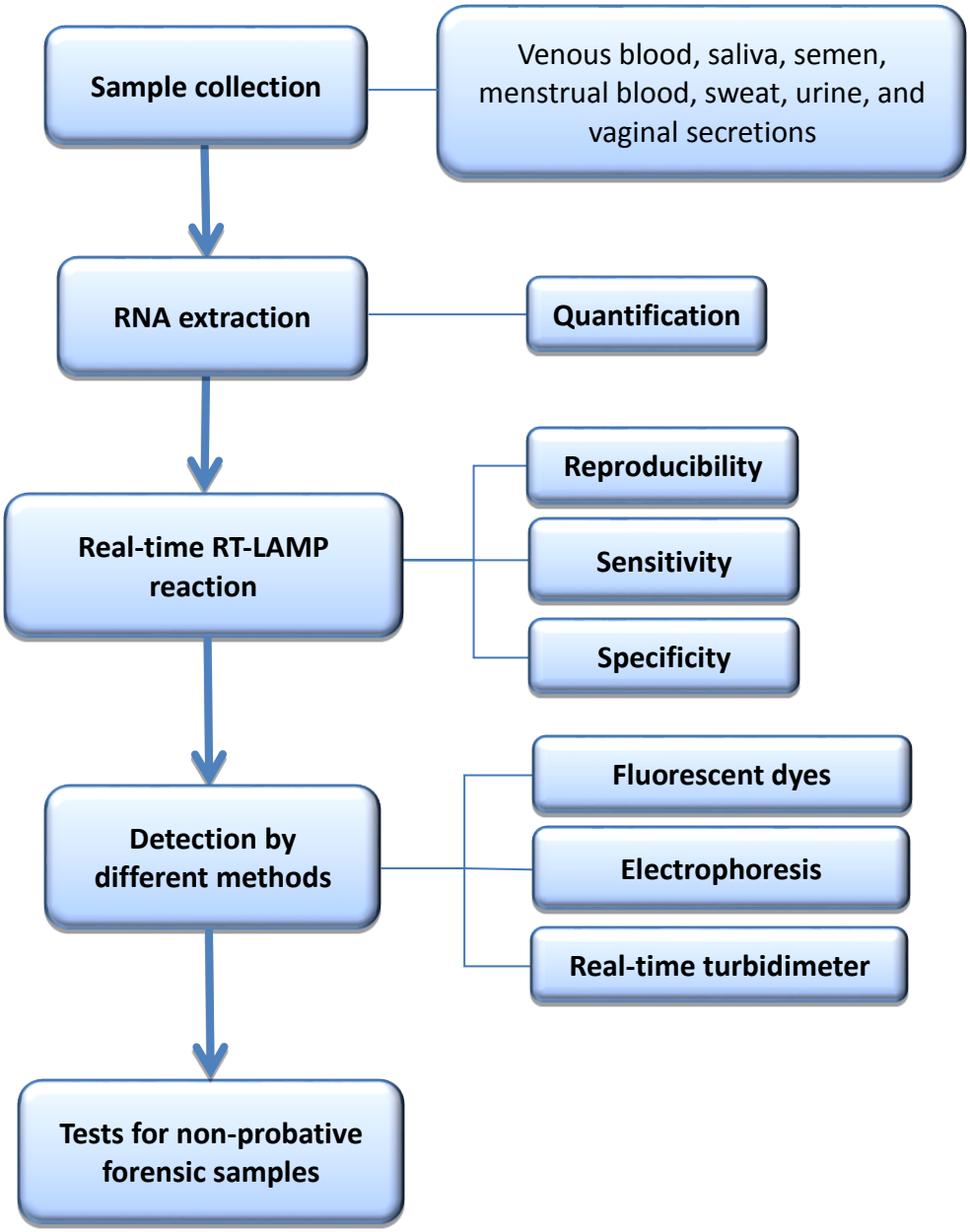


Figure 3-1 Flowchart of this study

3.2.1 Sample collection

The body fluids used in this study included venous blood, saliva, semen, menstrual blood, sweat, urine, and vaginal secretion. The samples were collected from six volunteers between 20 and 40 years old for each body fluid using procedures approved by Institutional Review Board (IRB) of Central Police University in Taiwan and National Research Ethics Service (REC reference number: 10/H0808/94, Appendix 1). Whole blood samples (10ml) were collected using phlebotomy procedure performed by qualified personnel in accordance with health and safety regulations. Freshly ejaculated semen samples were collected by the volunteers with a sterile plastic container. The samples were then used immediately or stored at -20°C. For the saliva samples, volunteers were asked to rinse their mouth in advance and saliva (2ml) was collected in a sterile plastic container. Menstrual blood was collected from a tampon from which the menstrual blood was squeezed into a collection tube. Sweat samples were collected with a sterile tube after the volunteers had exercised for 30 minutes. Urine samples were collected by volunteers with a sterile plastic tube after they woke up in the morning. Vaginal secretion samples were collected by volunteers with swabs and stored in a clean hood before RNA extraction.

Animal blood samples from eight species (pig, dog, chicken, cow, goose, goat, cat, and rabbit), used to detect cross-species reaction, were kindly provided by the Livestock Research Institute, Council of Agriculture, in Taiwan. The collection of samples from six species (pig, chicken, cow, goose, goat, and rabbit) was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC); samples of the other two species (dog and cat) were collected from the animal hospital with

appropriate informed consent from their owners. All the body fluids were immediately stored at -20°C after collection.

3.2.2 RNA and cDNA preparation

Total RNA was extracted from each body fluid with RNeasy® Mini Kit (Qiagen® Ltd, UK) according to the manufacturer's protocol (described in Section 2.1.1). For venous blood, saliva, semen, and menstrual blood, 50µl of each liquid sample was used for the extraction. For vaginal secretion, total RNA was extracted from swabs (approximately 1 cm^2). As the concentration of target materials (including epithelial cells and extracellular nucleic acids) in urine and sweat is much lower than that contained in the other body fluids, therefore both urine and sweat samples were concentrated from 10 ml to 1 ml before the extraction to obtain more starting materials. The extracted total RNA was then quantified (described in Section 2.1.2) and then used for RT-LAMP reaction or stored at -20°C immediately after extraction. For the 2-step LAMP, total RNA was reverse transcribed to cDNA (described in Section 2.1.3) immediately after extraction for further analysis.

3.2.3 Marker selection and primer design

Housekeeping genes are usually used as internal controls in studies on RNA. The result from the specific target can then be compared with the internal control or normalized with it. The most commonly used housekeeping genes are Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), Ubiquitin, *ß-actin* (*ACTB*), and 18S ribosomal RNA (*18S rRNA*). The *18S rRNA* forms part of the ribosomal small

component of eukaryotic ribosomes and is a widely used control for quantitative reverse transcription PCR because of its invariant expression across tissues and cells [280]. A comparison of specificity among *18S rRNA*, *ACTB,* and *GAPDH* showed that only *18S rRNA* could be detected in most samples, including blood, saliva, semen, menstrual blood, vaginal secretions, and skin tissues [148]. Hence, although the expression of *18S rRNA* cannot represent the total RNA, it is a reliable normalisation gene and is still one of the best choice to be used as an internal control [281]. In this study, *18S rRNA* was used as the internal control accordingly.

Haemoglobin involves in carrying oxygen from the lung to the peripheral tissues and returning carbon dioxide from tissues back to the lung via blood. It is important and essential in the red blood cell. The most common type of haemoglobin in adults is $\alpha_2\beta_2$ which comprises two alpha-globin (*HBA*) and two beta-globin (*HBB*). *HBB* has been reported to be expressed abundantly in blood [282]. Besides, from a collaborative exercise on mRNA profiling for the identification of blood organized by the European DNA Profiling Group, *HBB* was found to be the most abundantly detected mRNA in their result [147]. Thus, the mRNA transcribed from haemoglobin subunit beta (*HBB*) gene was chosen as the marker for blood in this study.

A set of four specific primers, including F3, B3, FIP and BIP primer, was designed for the LAMP reaction. DNA sequences of *18S rRNA* and *HBB* were extracted from GenBank (accession nos. U133 69.1 and NM 000518.4 respectively). During the preliminary tests, a series of primer sets designed by PRIMER EXPLORER V3 were evaluated for their amplification efficiency and specificity. The optimal primer sets for *18S rRNA* and *HBB* amplification were selected based on extensive trials. The respective sites of the selected primers are shown in Figure 3-2. The predicted length from F3 to B3c site for *18S rRNA* and *HBB* were 197 and 192 bp, respectively. FIP

primer for *HBB* was designed to span intron/exon junctions, shown as Figure 3-2(b) and Figure 3-3, to prevent the amplifications from any contaminating DNA. The sequences of primers are listed in Table 3-1.

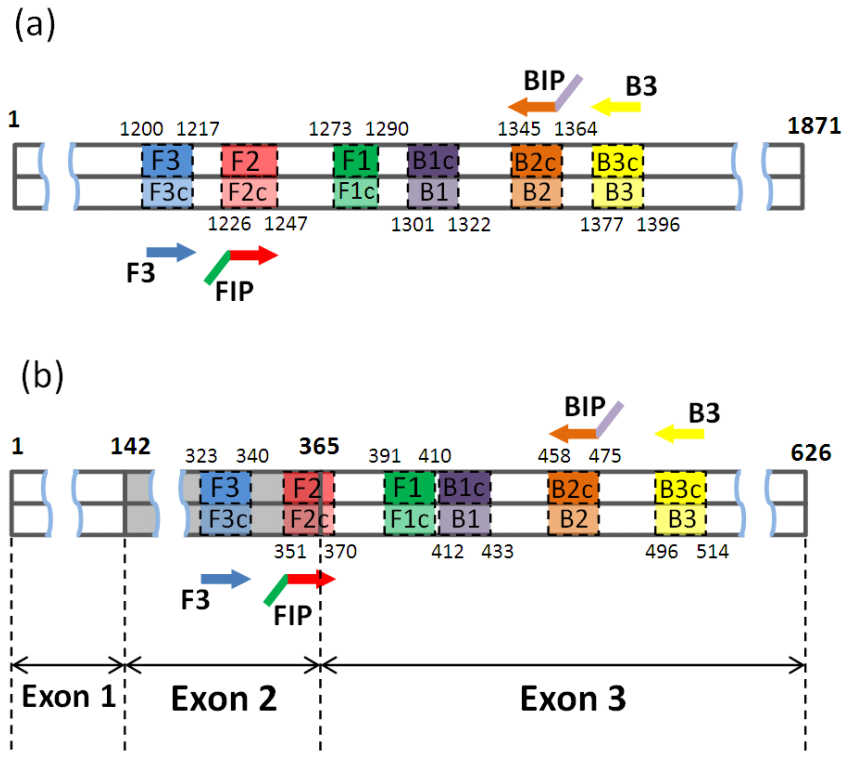


Figure 3-2 The sites of LAMP primers for *18S rRNA* (a) and HBB (b)

The nucleotide numbering starts at the 1st base of exon 1.

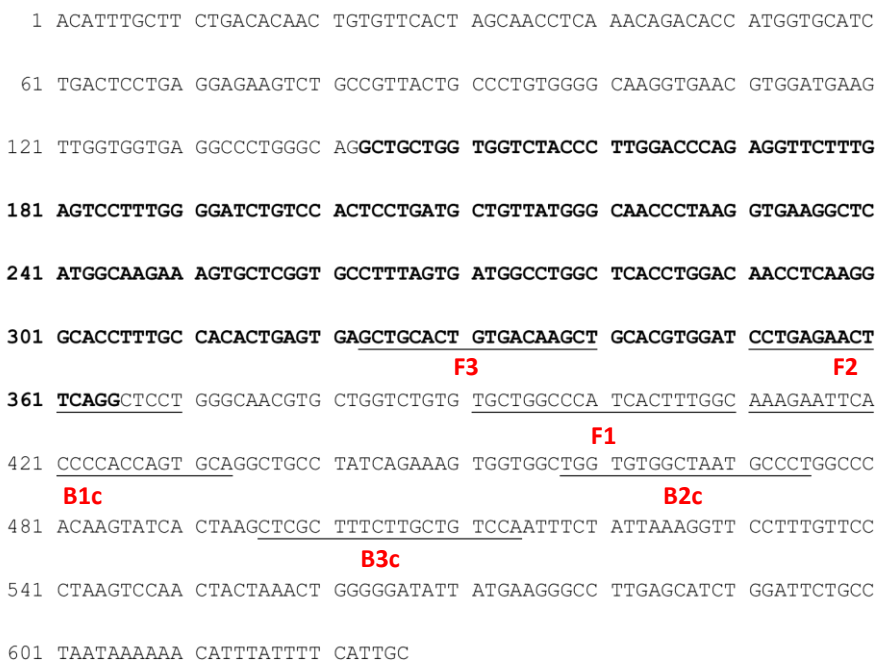


Figure 3-3 The nucleotide sequence of *HBB* (spliced) and locations of 6 specific sites

The specific sites, namely F3, F2, F1, B1c, B2c, and B3c, are underlined. To distinguish the different exons, the second exon is listed as bold face. The F2 region is designed in an intron-exon junction site.

Table 3-1 Information of the primers used in this study

Marker	Target	Primer	Sequence (5'-3')
18S rRNA	Housekeeping	F3	ATTGACGGAAGGGCACCA
		B3	TGCCAGAGTCTCGTTCGTTA
		FIP	CAATCCTGTCCGTGTCCGGGAGCCTGCGGCTTAATTTGAC
		BIP	AGCTCTTTCTCGATTCCGTGGGAGACAAATCGCTCCACCAAC
HBB	Blood	F3	GCTGCACTGTGACAAGCT
		B3	TGGACAGCAAGAAAGCGAG
		FIP	GCCAAAGTGATGGGCCAGCACCTGAGAACTTCAGGCTCCT
		BIP	AAGAATTCACCCCACCAGTGCAAGGGCATTAGCCACACCA

3.2.4 LAMP reaction and RT-LAMP reaction

LAMP with cDNA transcribed before the reaction was performed in the preliminary test. Complementary DNA was added to the total reagent (Table 3-2). The

whole reagent was heated at 95°C for 5 minutes and then chilled on ice immediately. Eight units of *Bst* DNA polymerase (8U/µl) was then added in each tube for the reaction and the final volume for the whole LAMP reaction was 25µl. Finally, LAMP reaction was carried out with the following conditions: incubating at 65°C for 60 minutes in the cycling amplification and 80°C for 10 minutes to terminate the reaction. The reaction was performed with the thermal cycler GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA).

Table 3-2 Components of the reagents for the LAMP reaction

Reagents	Volume
5M Betaine	5µl
5uM F3 primer	1µl
5uM B3 primer	1µl
20uM FIP primer	1µl
20uM BIP primer	1µl
0.25M $(NH_4)_2SO_4$	1µl
0.5M Tris-HCl (pH8.25)	1µl
0.25 KCl	1µl
25% Triton X-100	1µl
40mM dNTP	1µl
cDNA	10µl
Total	24µl

After the preliminary test, all samples were tested with one-step RT-LAMP using Loopamp RNA Amplification Kit (Eiken Chemical Co. Ltd., Tochigi, Japan) and following the manufacturer's protocol (see Section 2.4.2.2). The total volume for the entire reaction was 25µl which consisted of 40µM for each inner primer FIP and BIP,

5µM for each outer primer F3 and B3, 12.5µl reaction mix, 1U enzyme and 5µl RNA template (extracted from 50µl body fluids).

The LAMP product was detected by different methods including electrophoresis, fluorescence, and real-time turbidity measurement. An aliquot (1.5µl) was added to loading buffer and electrophoresis was performed with the 2 % agarose gel and SYBR Green in 1X TBE buffer at 120V and 400mA for 40 minutes. The gel image was then photographed under UV transillumination (Syngene® GBox, Maryland, USA) using Genesnap® from Syngene® image acquisition software (Vision-Capt version 14.2). For fluorescence detection, calcein was added in the LAMP reagent before the reaction (see Section 2.4.3). For the real-time turbidity measurement, the reaction was performed by using the real-time turbidimeter LA-500 (Eiken Chemical Co. Ltd., Tochigi, Japan).

3.2.5 Preliminary tests

Preliminary tests, including RNA quantification and optimized condition of LAMP reaction, were performed in advance. RNA was extracted from 3 samples for each body fluid and each extracted RNA was then quantified in triplicate to evaluate the method of RNA extraction and the difference of the quantification of RNA extracted from different body fluids.

Preliminary tests of both two-step LAMP and RT-LAMP were performed with RNA extracted from blood, semen, saliva, and negative control RNA. Several primer sets of *18S rRNA* and *HBB* markers were tested separately to evaluate the reaction conditions for RT-LAMP, including the temperature of the reaction (60-65°C), requirement of primers (inner primers and outer primers).

3.2.6 Reproducibility, specificity, and sensitivity analyses of real-time RT-LAMP

Real-time RT-LAMP reaction was performed in triplicate with RNA extracted from each body fluid to evaluate its reproducibility. Real-time RT-LAMP reaction with RNA extracted from 6 different individuals for each body fluid was also performed to estimate the specificity of LAMP. Besides, real-time RT-LAMP reaction was also performed with total RNA extracted from blood samples of the 8 animal species mentioned above (with triplicate samples of each species) to detect cross-reaction with other animal species. Total RNA from venous blood was serially diluted in triplicate using a 10-fold dilution series down to a final dilution of 1 to 10^{-7} ng RNA for the real-time RT-LAMP to evaluate the sensitivity and limit of LAMP.

3.2.7 Cross-reaction test of real-time RT-LAMP

To determine the limit of detection within a mixture of body fluids, real-time RT-LAMP was also performed with mixed RNA extracted from blood and from the other body fluids (including semen, saliva, sweat, urine, and vaginal secretion) in different proportions (9:1, 1:1, and 1:9) separately to check the cross-reaction between different body fluids. Menstrual blood was not included in this test because it originally contains blood.

3.2.8 Application on non-probative forensic trace blood samples

To evaluate the potential of RT-LAMP on forensic blood identification, non-probative forensic samples were collected and tested in this study. All the samples were screened by presumptive blood tests and total RNA was extracted as the materials for blood identification by RT-LAMP following the conditions provided above.

3.2.8.1 Sample preparation

Twenty-one non-probative forensic trace blood samples were collected (the real casework traces are explained in Table 3-3). The time since deposition of these samples ranged from 1 month to 12 years. All blood was transferred with sterile cotton swabs and stored appropriately to preserve its integrity as best as possible for further analysis. Total RNA was extracted from the samples of approximately 1 cm^2 materials (swab, fabric or gauze) from a blood stained area, and 5µl of each was used for real-time RT-LAMP.

Table 3-3 Real casework traces

Forensic sample	Sample type	Time interval between sampling at the crime scenes and analyzing in this study	Sample locations at the crime scenes
1	Swab	3 months	Mobile phone
2	Swab	3 months	Floor
3	Swab	2 months	Bed
4	Swab	2 months	Chassis of a bus
5	Swab	1 month	Edge of a knife
6	Swab	1 month	Edge of a meat
7	Swab	2 months	Edge of a knife
8	Swab	2 months	Knife handle
9	Swab	2 months	Chassis of a bus
10	Swab	1 month	Front console box of
11	Swab	2 months	External vaginal swab
12	Swab	2 months	Cervical swab
13	Fabric	2 months	Underpants
14	Swab	1 month	External vaginal swab
15	Swab	10 months	Cervical swab
16	Swab	1 month	Cervical swab
17	Fabric	3 months	Trouser pocket
18	Fabric	1 month	Trouser pocket
19	Fabric	1 month	Jacket
20	Gauze	12 years	Blood stain
21	Gauze	12 years	Blood stain

3.2.8.2 Presumptive tests

All the samples were screened by presumptive blood tests, including Kastle-Meyer test [255] and HemDirect Hemoglobin test (SERATEC®, Göttingen, Germany)

[256], following procedures described in Section 2.5.2. HemDirect test was not analysed in some of these samples following the standard operating procedure of the laboratory.

3.2.8.3 DNA and RNA preparation

DNA was extracted from these blood samples by QIAamp® DNA Mini Kit (described in Section 2.5.3) and quantified using 7500 Real-Time PCR System with Quantifiler™ Human DNA Quantification Kit (described in Section 2.5.4). Total RNA was extracted with RNeasy® Mini Kit as mentioned in Section 2.1.1 and quantified with NanoDrop® ND-1000 UV spectrophotometer (described in Section 2.1.2).

3.2.8.4 DNA profiling

DNA profiling was performed using AmpFLSTR® Identifiler® PCR Amplification Kit (described in Section 2.5.5.1). The condition of capillary electrophoresis is described in Section 2.5.5.2.

3.3 Results and discussion

3.3.1 Preliminary tests

3.3.1.1 RNA extraction and quantification

In the preliminary tests of RNA extraction and quantification, RNA was extracted from 3 samples of each body fluid. Each RNA was quantified in triplicate using Nanodrop®. The original quantification data is listed in Appendix 2 and a comparison of concentration for each body fluid was shown as Figure 3-4. The result showed that semen samples yielded high quantity of RNA (47.4±10.3 ng/µl from 50µl liquid sample) while sweat yielded a smaller amount of RNA (6.9±2.1 ng/µl from 50µl concentrate which was concentrated from 500µl liquid sweat).

It is important to perform the experiment with the same starting amount of materials in order to compare the result of different body fluids. However, the efficiency of RNA extraction results in the difference of yields. Meanwhile, the main source of RNA varies from body fluid to body fluid (see Section 1.3.3), so the quantity of RNA extracted from different body fluids varies accordingly. Besides, other individual factors, including physiological or psychological conditions, may also affect the extraction [283]. The proportion of the target mRNA also varies among the total RNA. Thus, instead of using the same amount of samples, approximately 20ng of the total RNA was set as a controlled amount to eliminate variety of the results caused by efficiency of RNA extraction and inconsistent expression of RNA in this study. The limitation of the test also can be calculated with the values of the sensitivity and the quantity of RNA extracts in this study.

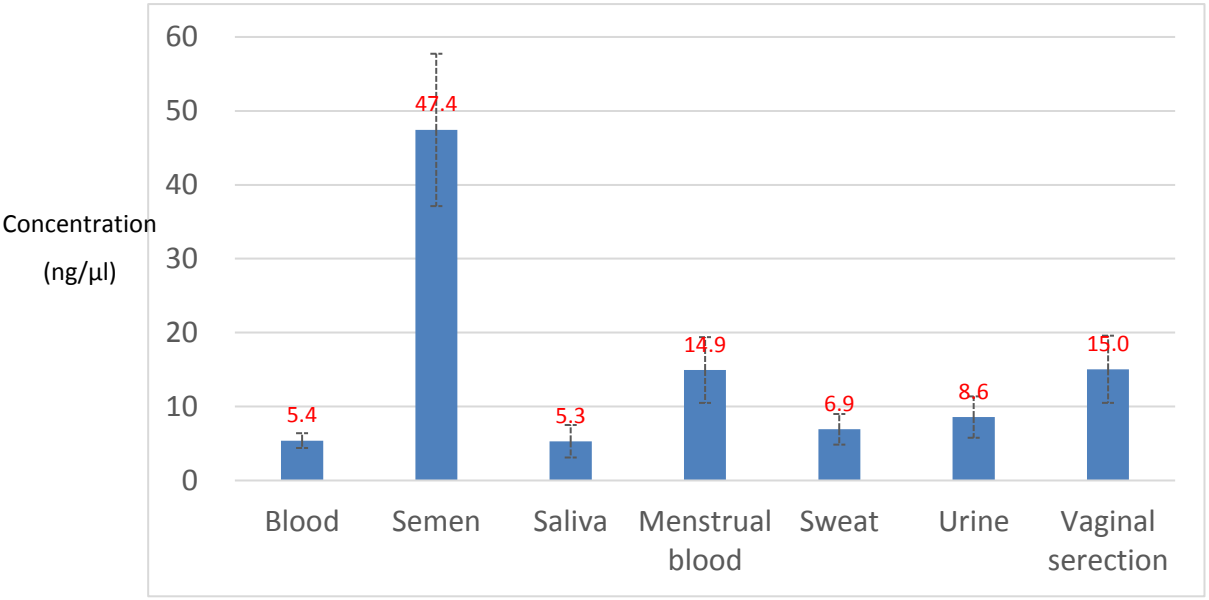


Figure 3-4 RNA yields of each body fluid

* Total RNA was extracted from samples concentrated from 10ml to 1ml before the extraction to obtain more starting materials.

3.3.1.2 Two-step LAMP

To evaluate the potential of LAMP, two-step LAMP (reverse transcription and LAMP reaction were carried out separately) was tested at the beginning of the preliminary tests. Two-step LAMP was tested with primer sets of the *HBB* marker and gel electrophoresis. RNA extracted from blood, semen, and saliva was used for this preliminary test. Negative control sample was added in each step, including RNA extraction, reverse transcription, and LAMP reaction.

The electrophoresis result of LAMP is shown as Figure 3-5. Significant LAMP products could be found in 3 blood samples. There was no band in semen, saliva samples or each negative control. The result revealed the possibility of blood identification using LAMP reaction and the specificity of *HBB* marker in LAMP reaction.

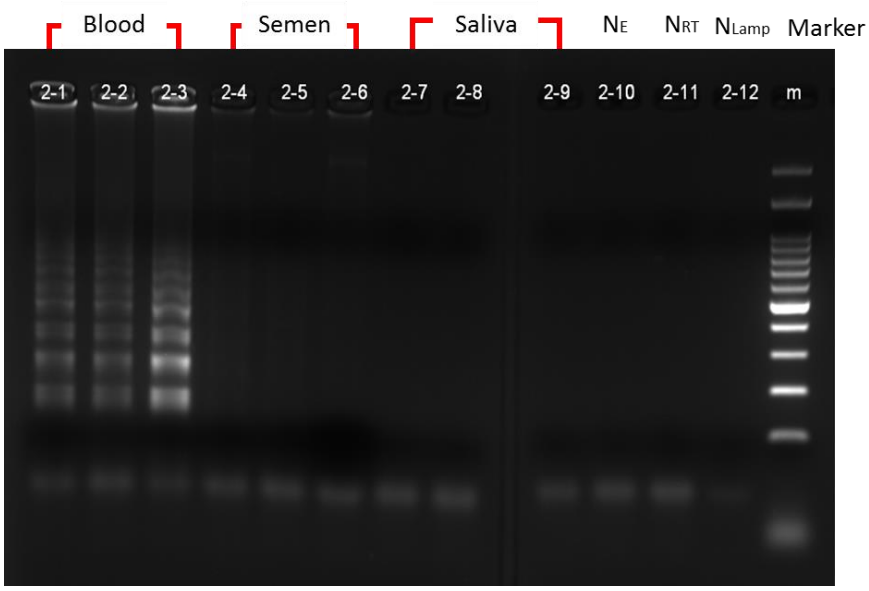


Figure 3-5 The result of two-step LAMP in *HBB* marker

Lane 2-1, 2-2 and 2-3: LAMP products from 3 different blood samples.
Lane 2-4, 2-5 and 2-6: LAMP products from 3 different semen samples.
LAMP 2-7, 2-8 and 2-9: LAMP products from 3 different saliva samples.
Lane 2-10 (N_E): Negative control for RNA extraction.
Lane 2-11 (N_{RT}): Negative control for reverse transcription.
Lane 2-12 (N_{LAMP}): Negative control for LAMP.
m: Marker.

LAMP reaction with/without inner primers (FIB and BIP) was also tested in the preliminary test (Figure 3-6). The result showed that LAMP products could be obtained when the whole reaction performed with all inner and outer primers. However, no amplification of LAMP products was observed when the reagents contained only outer primers (F3 and B3). It meant that inner primers are required in the whole reaction as predicted since they act as a self-priming role and no chain reaction can be formed without it. The result is also consistent with the study done by Notomi *et al.* in 2000 [198].

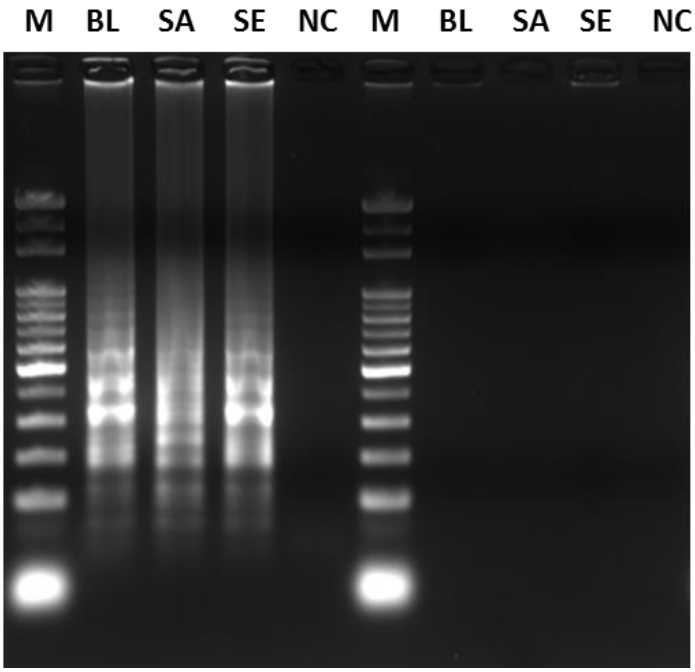


Figure 3-6 LAMP reaction (*18S rRNA*) with/without FIP and BIP

Lane 1 and 6 (M): Marker.
Lane 2 and 7 (BL): Blood sample.
Lane 3 and 8 (SA): Saliva sample.
Lane 4 and 9 (SE): Semen sample.
Lane 5 and 10 (NC): Negative control.

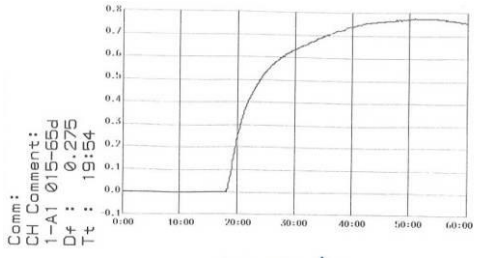
3.3.1.3 RT-LAMP and optimizing condition

In the preliminary test, primer sets for *18S rRNA* marker were chosen and tested to obtain optimized condition of RT-LAMP reaction. In the beginning, *18S rRNA* marker was tested with blood and negative control at 60°C within 60 minutes. LAMP product increased quickly at 19.9 to 21.8 minutes (Figure 3-7). However, the LAMP product of negative control started to accumulate rapidly around 49 minutes (Figure 3-8). The reactions were repeated several times, all with similar results. In other words,

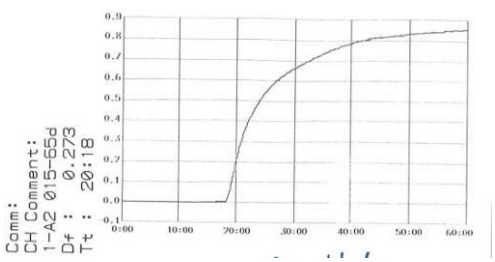
LAMP products could be found in both positive and negative samples in such thermal condition in the preliminary test.

Non-specific amplification of LAMP products may be caused by several reasons, such as the concentration of primers, temperature of the reaction, GC contents of primer, and the formation of secondary structures [284]. Although the threshold time (Tt) of positive reactions can be distinguished from negative ones (21.8 to 49 minutes), it would be difficult to analyse the data if Tt is at the edge of a cut-off value.

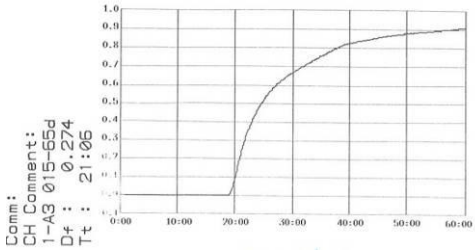
To overcome this problem, several primer sets for *18S rRNA* marker with the same concentration of primers were tested to obtain the best candidate. The final optimized primer sets are listed in Table 3-1. Besides, different reaction temperatures were also tested (60-65°C). When the temperature was set as 65°C, LAMP products were detected within 60 minutes in all samples except negative control with *18S rRNA* marker. For *HBB* marker, only blood and menstrual blood showed positive result at 65°C (see Section 3.3.2). However, the Tt value of all samples for both markers was delayed as well. Since LAMP is an isothermal reaction, primer annealing is carried out at the same temperature. Primers cannot bind to the template easily if the annealing temperature is too high. Thus, raising the temperature increases the specificity but decreases the efficiency of reaction on the other hand. In this study, increasing the specificity of the LAMP reaction is needed. Thus, even with the defect of lowering amplification efficiency, the temperature for the following LAMP experiments was set as 65°C and the cut-off time for the test was set as 60 minutes.



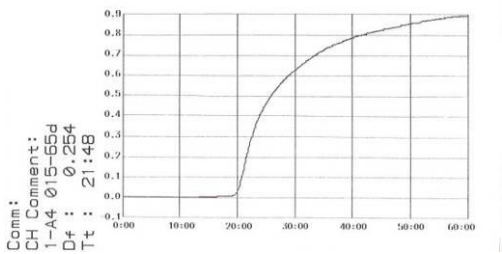
Blood sample 1 (*18S rRNA*)



Blood sample 2 (*18S rRNA*)

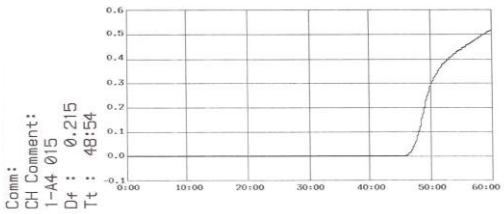


Blood sample 3 (*18S rRNA*)



Blood sample 4 (*18S rRNA*)

Figure 3-7 Amplification curves of RT-LAMP positive product with *18S rRNA* marker by using real-time turbidimeter



Negative (*18S rRNA*)

Figure 3-8 Amplification curve of RT-LAMP negative product of *18S rRNA* marker by using the real-time turbidimeter

3.3.2 Reproducibility and specificity analysis of real-time RT-LAMP

Subsequent to determining the optimal amplification conditions, the reproducibility and specificity of real-time RT-LAMP were analyzed in triplicate for

both loci using one sample of each body fluid. The average and standard deviation of Tt (min) are shown in Table 3-4 (original data is listed in Appendix 3). The RT-LAMP products from the *18S rRNA* locus were detected in all tested body fluids as expected. The Tt ranged from 36.0±0.9 to 42.8±1.3 minutes. *HBB* LAMP products were only detected in venous and menstrual blood within 33.1±4.8 and 40.6±3.3 minutes respectively, which was as expected if the method was specific to blood. No amplification products were observed for the negative control (NC). The results indicated that the repeat tests were reproducible in the triplicate assay and specificity for LAMP reaction was observed as expected.

Table 3-4 Analysis of the expressions for *18S rRNA* and *HBB* performed in triplicate using one sample from each body fluid using real-time RT-LAMP

Gene	Venous blood	Saliva	Semen	Menstrual blood	Sweat	Urine	Vaginal secretions	NC*
18S rRNA	40.7±2.2	36.0±0.9	41.0±2.0	39.1±2.3	42.8±1.3	41.6±2.1	43.0±0.9	-
HBB	33.1±4.8	-	-	40.6±3.3	-	-	-	-

The average and standard deviation of Tt (min) were from the analysis in triplicate

* NC represents the negative control (without RNA template), and the symbol "-" is for no RT-LAMP products detected

Additionally, the expression of these two markers was analyzed for all body fluids collected from six individuals for each of the body fluids. A positive result (within 60 minutes) was observed in all samples from the *18S rRNA* locus (Figure 3-9, original data is listed in Appendix 3), and the Tt value (min) ranged from 40.9±5.0 (for menstrual blood) to 51.1±5.7 (for sweat). However, *HBB* expression was only detected in the venous (36.8±4.1 minutes) and menstrual blood (46.6±5.9 minutes), and the urine samples from two females (50.6 and 53.3 minutes). It was confirmed subsequently that these urine samples were collected during menstruation and

therefore most likely mixed with their menstrual blood. The result showed that some body fluids, such as urine and menstrual blood in this test, may be mixed and contaminated with each other. On the other hand, the result also represented that LAMP is so sensitive that even minute quantity of blood can still be detected. This result further illustrated the reproducibility and specificity of these markers though individual variation was observed.

Figure 3-9 Analysis of the expressions for *18S rRNA* and *HBB*

The reaction was performed by the real-time RT-LAMP in different body fluids from 6 individuals for each of the body fluids. The symbol of "*" represented the Tt (50.6 and 53.3 minutes) for *HBB* of urine samples from the two females during the menstrual period.

To detect any cross-reaction with other animal species, total RNA was extracted from the blood of eight animal species (triplicate samples were collected for each of the species). The accumulation of LAMP product from the reaction with human blood samples reached the threshold within 37.5 minutes. No *HBB* LAMP products were detected within 60 minutes of the reaction (the cut-off time set in the study, Figure 3-10) except that a reaction product from one of the pig blood samples (not for the other two) was detected after 70.5 minutes. Nevertheless, as it was 10 minutes

after the cut-off time, it was still confirmed as negative. The result showed no cross reaction of human blood with these animal species and therefore, the specificity was further confirmed. However, blood from more species, especially the species which are more closely related to human being such as chimpanzees, should be tested as well.

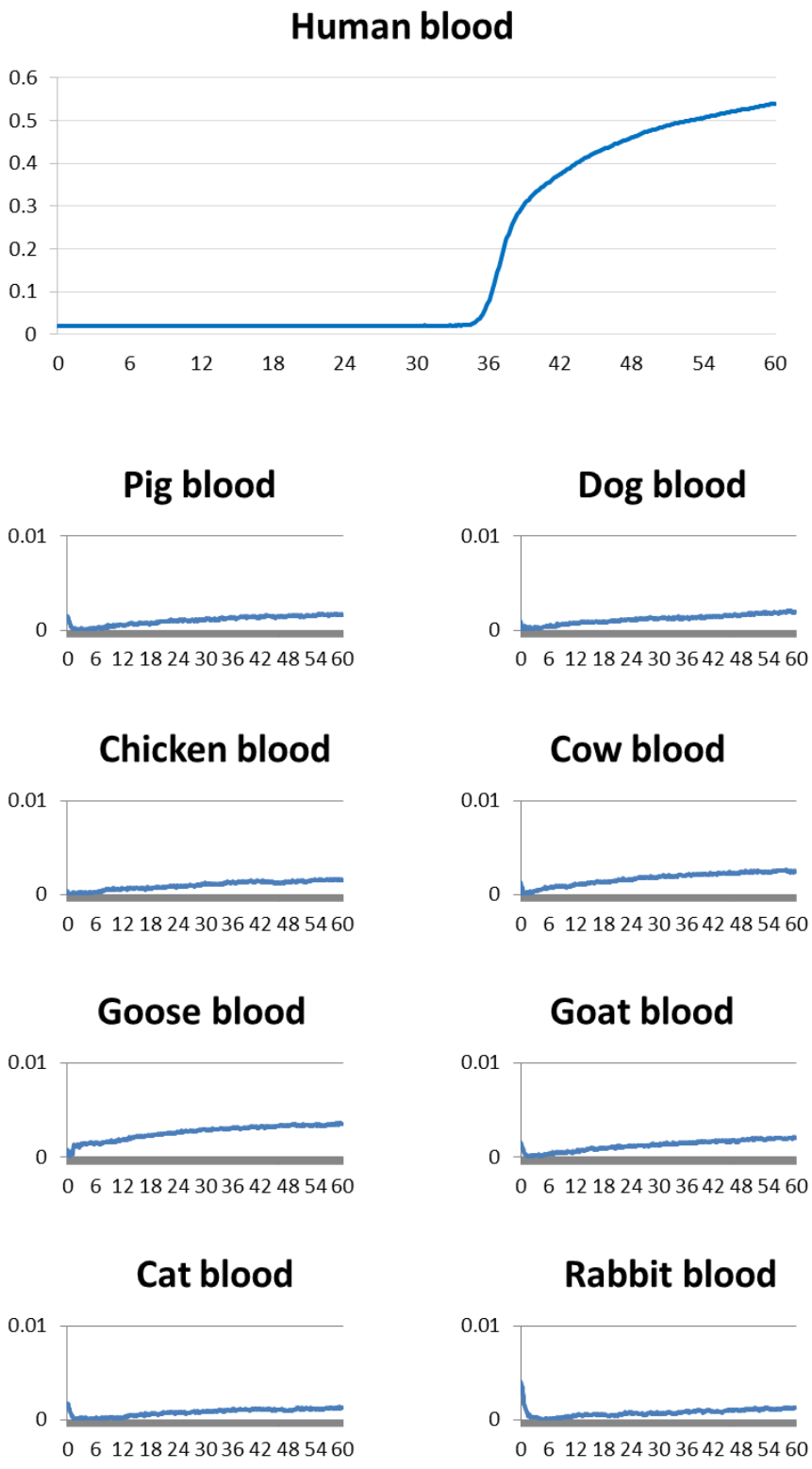


Figure 3-10 The result of RT-LAMP for the RNA of animal bloods

The data was normalized by the minimum value of the absorption. The Tt value for the human blood in this test was 37.5 minutes. No amplification was found in the blood samples of 8 animals within 60 minutes (The figure only shows one sample for each animal). The x-axis and y-axis of the amplification curve represent the reaction times (min) and absorbance respectively.

3.3.3 Sensitivity

To determine the limit of detection of the test, total RNA from venous blood was serially diluted in triplicate using a 10-fold dilution series down to a final dilution of 1 to 10^{-7} ng total RNA. Products for both the *18S rRNA* and *HBB* loci were observed from a minimum of 10^{-5} ng total RNA by real-time RT-LAMP within 60 minutes (Figure 3-11), and the Tt values were 57.3±3.5 and 56.5±1 minutes respectively.

LAMP was reported to offer potential quantitative molecular analysis [285]. In this study, the r^2 value of the standard curves (Figure 3-11) reached 0.995 for both markers. The r^2 value measures the closeness of fit between the standard curve and the Tt values of the total RNA used in the assay. A value of 1 indicates a perfect fit between the data points and the curve. Thus, the result showed that LAMP is not only able to identify body fluids but also capable of quantifying target nucleic acid, which can further be used to estimate the amount of the body fluids.

The average concentration of total RNA extracted from 50µl blood samples was 5.4 ± 1.0 ng/µl in a final elution volume of 30µl; the limit of detection was 10^{-5} ng total RNA, which was equivalent to 10^7X dilution of blood. It follows that traces of blood can be detected and quantified, well below the amounts required for successful DNA profiling.

There have been many tests conducted on the sensitivity of presumptive tests of blood but the results contradict one another [286]. Among the current methods used in blood identification (see Section 1.1.1), the KM test is extremely sensitive and routinely used as a presumptive blood test in forensic practice. The sensitivity of the KM test ranges from 10^4X to 10^7X dilution [19, 21, 286]. Vennemann *et al.* proposed that the KM test only showed 54.4% positive results at 10^7X dilution in their study [19].

Accordingly, blood identification by LAMP proposed in this study is considered at the same level or even better than the KM test.

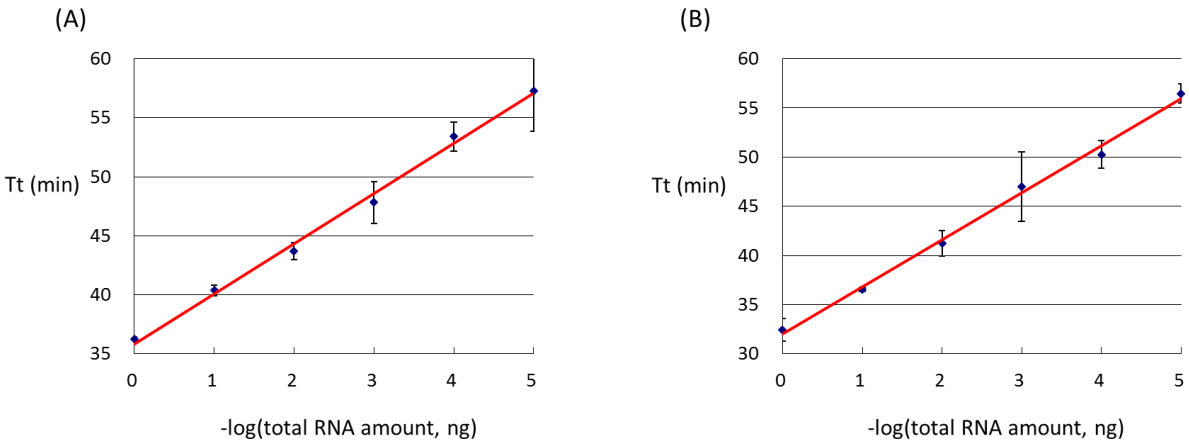


Figure 3-11 Sensitivity test of *18S rRNA* (A) and *HBB* (B) by the real-time RT-LAMP

The test was performed in triplicate. The r^2 of both curves was 0.995.

These data illustrated the relatively high sensitivity of this assay. It is indicated that real-time LAMP permits a quantitative analysis of minute amounts of nucleic acids according to the study represented by Mori *et al* [225]. However, the total RNA amount was used as a reference amount in this test. The amount of *18S rRNA* for each body fluid from the same amount of total RNA varies, so the standard curve may not the same if total RNA extracted from other body fluids was used. Besides, since the level of RNA expression may vary in different individuals as well as tissues, the standard curve obtained from different individuals or tissues may vary as well. Given a body fluid found at a crime scene, it is more important to know what kind of body fluid stain it is than how much it is. Thus, usually qualitative analysis is more useful than quantitative analysis in forensic science. From the result of the sensitivity test, LAMP has high potential to be used as a tool for blood identification. Nevertheless, the quantification of RNA (*HBB*) may be still useful.

3.3.4 Test of LAMP in mixtures

Additionally, to determine the limit of detection within a mixture of body fluids, total RNA from venous blood was mixed with RNA extracted from one of the other tested body fluids (semen, saliva, sweat, urine, and vaginal secretions) at different ratios of 9:1, 1:1 and 1:9. The amount of total RNA for each mixture was approximately 20ng. The expression of both *18S rRNA* and *HBB* genes was observed for all mixture preparations (Figure 3-12) with similar amplification pattern for each gene. These results indicated no significant influence on the detection of blood using *HBB* marker within a mixed sample.

The accumulation of LAMP products from *HBB* marker reached the threshold before 31 minutes while it was more than 40 minutes for *18S rRNA* marker. Meanwhile, the Tt value from *HBB* marker for each set of mixtures increased when the amount of total RNA from blood reduced in the mixture. These findings are consistent with previous results.

Although no cross-reaction was observed in this test, this is just a basic test for the effect of mixture. Body fluids may be mixed with the other fluids which were not tested here, such as nasal mucus. Besides, mixtures of body fluids may contain more than two body fluids. For example, menstrual blood may contain vaginal secretions, blood, urine, and even sweat. More tests for the mixtures with the other body fluids and with more than two fluids should be carried out before a practical use for forensic investigation.

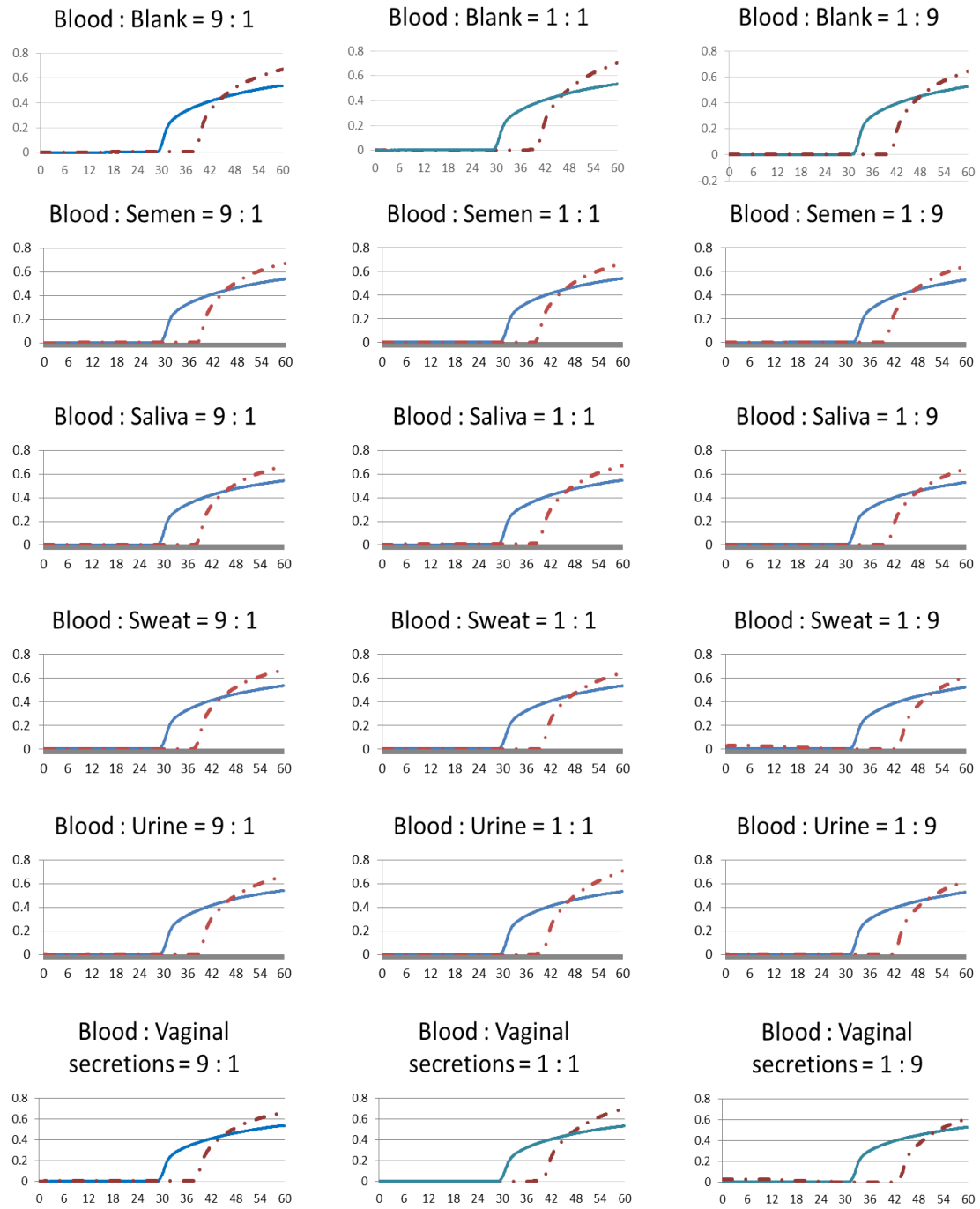


Figure 3-12 Sensitivity test of the venous blood for *18S rRNA* (dashed lines) and *HBB* (bold lines) in the mixture

Combination of the reaction curves for *18S rRNA* and *HBB* is shown in this figure (only one sample of each mixture is shown here). The x-axis and y-axis of the amplification curve represent the reaction times (min) and absorbance respectively.

LAMP has been proven to be a new tool for DNA quantification [287]. However, for the following reasons, it may not be realistic to quantify body fluid using LAMP, not to mention quantifying the mixed body fluids. First, the efficiencies of RNA extraction, reverse transcription, and LAMP reaction (RT-LAMP) all impact the yield of cDNA and LAMP products, which makes the quantification inaccurate easily. Second, the amount of the target marker (*HBB* mRNA in this study) cannot reflect the quantity of the target body fluid since its expression may vary among individuals on the one hand, and illegitimate transcription of target mRNA may occur on the other hand. Accordingly, it is hard to establish a standard correlation between the target mRNA and body fluid with this assay.

3.3.5 Detection of RT-LAMP products by electrophoresis and fluorescence

To examine the alternative means of detecting RT-LAMP products, separation of products on an agarose gel and monitoring the presence of fluorescence for calcein were used. The ladder-like structure on agarose gel, and calcein fluorescence, was observed for *18S rRNA* in all tested body fluids (Figure 3-13). *HBB* products were only recorded with the venous and menstrual blood. These results were found to be concordant with the detection of the RT-LAMP products using a turbidimeter (real-time RT-LAMP).

Monitoring the presence of fluorescence could be performed remotely from the laboratory, such as at a crime scene. Since speed is of great importance in crime scene investigations, the goal is to develop a cheap, reliable, and one-tube test that could be used at the crime scene to not only determine the body fluid type but also

be human specific. This would enable the fast tracking of samples for DNA profiling and provide more information for the investigation on site. Detection of RT-LAMP products with calcein provides advantages over PCR such as simplicity and speed. Thus, it shows high potential of RT-LAMP for forensic body fluid identification.

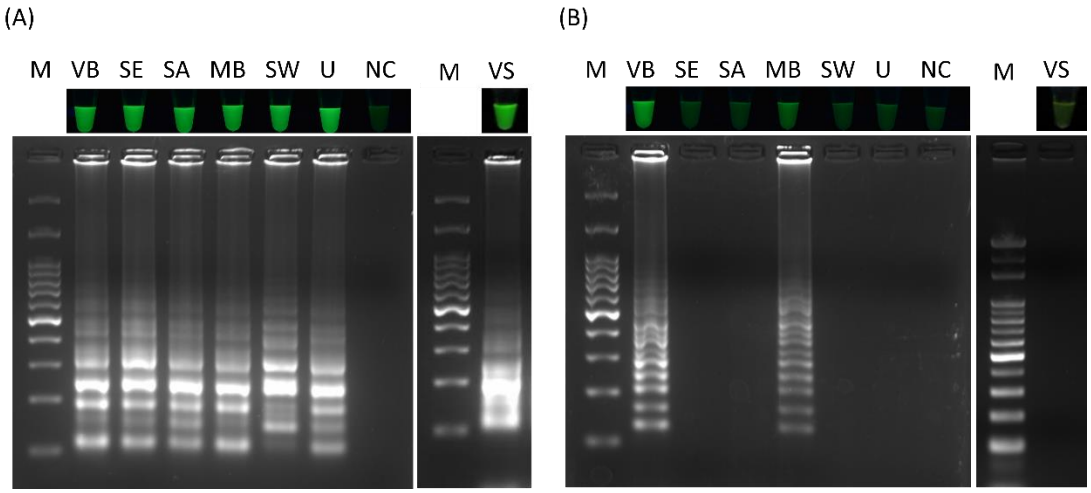


Figure 3-13 Detection of LAMP products for *18S rRNA* (A) and *HBB* (B) by agarose gel electrophoresis and calcein fluorescence

M: 100bp DNA marker; VB: Venous Blood; SE: Semen, SA: Saliva; MB; Menstrual Blood; SW: Sweat; U: Urine; VS: Vaginal Secretions; NC: Negative Control. Conditions of the photography for the calcein fluorescence are ISO100, F/8 and 1/8 second for exposure.

3.3.6 Applications on the non-probative forensic trace blood samples

The real-time RT-LAMP system was applied to the identification of body fluids collected from 21 non-probative forensic samples where it was suspected that the blood was present. The real casework traces are described in Table 3-3. The time intervals between sampling at the crime scene and analysis in this study ranged from about 1 month to 12 years. The original blood samples were observed on the surface of the substrates such as the mobile phone, bed, knife, trouser pocket, and so on.

All samples except Sample 6 tested positive for the presence of blood using KM and/or HemDirect. For all these samples, expression of the *18S rRNA* and *HBB* was detected within 60 minutes (Table 3-5), thus confirming the presence of blood. For Sample 4, 5 and 6, results of the presumptive tests (KM or HemDirect tests) were either not concordant or unclear. Nevertheless, the presence of blood was confirmed using the real-time RT-LAMP assay. An example is Sample 6, which was collected from a meat cleaver allegedly used as a weapon in an assault. It was suspected that the cleaver was subsequently washed/cleaned. Forensic STR profiling from the blade of the cleaver matched the suspect using 16 loci (15 STR and the Amelogenin) lending support (and did other relevant information) that the DNA profile was from an area of the cleaver that had been bloodstained, but the presence of blood was removed by washing. Our data indicated that the real-time RT-LAMP exhibits greater sensitivity than the KM and HemDirect tests on blood identification.

Besides, some studies demonstrated that reliable mRNA may be obtained from post-mortem tissues especially in dried or frozen samples, where mRNA degradation is slower [124, 125]. Other studies showed that it is possible to get successful mRNA profiling in 16-23 year-old bloodstains by reverse transcription and real-time PCR [126, 127]. In this study, two old bloodstains (stored in envelopes for 12 years) were also tested as positive, which proved that LAMP is capable of identifying old body fluid stains dating back up to 12 years.

Table 3-5 Identification of the non-probative forensic samples

Forensic sample	KM[a]	HemDirect[a]	$Tt_{18S\ rRNA}$ (min)	Tt_{HBB} (min)	Number of Successfully amplified loci in the DNA profile[c]
1	+	+	47.3	53.9	16/16
2	+	+	41.9	46.6	16/16
3	+	+	45.0	54.6	14/16
4	+	-	46.2	50.5	16/16
5	±	+	46.9	51.1	0/16
6	-	NA[b]	48.5	51.0	16/16
7	+	+	40.3	50.2	16/16
8	+	+	40.4	41.5	0/16
9	+	+	46.0	51.6	8/16
10	+	+	44.3	46.6	4/16
11	+	NA[b]	41.4	24.4	16/16
12	+	NA[b]	38.3	23.1	16/16
13	+	NA[b]	38.9	46.4	16/16
14	+	NA[b]	36.2	29.4	16/16
15	+	NA[b]	41.7	25.1	16/16
16	+	NA[b]	38.3	22.9	16/16
17	+	NA[b]	49.4	37.3	16/16
18	+	NA[b]	59.7	29.8	16/16
19	+	NA[b]	53.6	35.2	16/16
20	+	NA[b]	52.1	42.9	16/16
21	+	NA[b]	53.5	51.1	16/16

[a] The symbols of "+" and "-" represent the positive and negative results respectively, and "±" as the weak positive result.

[b] HemDirect test was not analysed (NA) in this sample following the standard operating procedure of the laboratory.

[c] DNA profiling was performed with AmpFlSTR® Identifiler® PCR Amplification Kit, which contains 16 loci including a gender-determining marker (Amelogenin). "16/16" represents a full profile and "0/16" represents none of loci was obtained. The other symbols represent partial profiles (the partial profiles are listed in Appendix 4).

3.4 Conclusion

Body fluid identification is highly relevant to forensic casework. The unequivocal identification of the cellular origin of crime scene evidence used for DNA profiling can provide crucial information for crime scene investigation. Meanwhile, RNA analysis has become a reliable method in forensic investigations. There have been a number of studies concerning mRNA or miRNA assays for body fluid identification. Typically, the tissue-specific marker is either detected by real-time PCR or by capillary electrophoresis. These methods have shown an increase in sensitivity of the test compared to previous methods and a real potential in forensic practice. By comparison with the RT-LAMP used in this study, however, these methods are time-consuming and require multiple steps, risking potential loss of sample. Furthermore, the relative success of a multiplex mRNA-profiling system for the forensic identification of body fluids can often be unbalanced due to unequal expression of the different RNAs in a tissue [148]; though in general miRNAs are extremely conservative across species, however, some human miRNA are not human-specific [288]. It is also observed that the body fluid-specificities of some identified miRNAs exhibit reported inconsistencies [5]. These are the reasons to explore an alternative method for body fluid typing.

LAMP was introduced for rapid detection of blood in this study. The optimized primers and LAMP conditions were observed for the LAMP reaction of *18S rRNA* and *HBB* markers. The same amount of total RNA (20ng) was set as a control to eliminate variety of the results. Based on the standard curve of sensitivity test, the amount of total RNA can be deduced with the Tt value. The amount of the sample can be further calculated with the result of RNA extraction and quantification in the preliminary tests.

Although it is arbitrary to estimate the amount of the sample, it still can be used as a clue for further forensic investigation.

Body fluids may be mixed with each other regularly or accidentally. For example, Female urine may contain vaginal secretions. Menstrual blood may contain vaginal secretions, blood, and urine. For forensic examinations, vaginal swabs collected from a victim of a sexual assault case may contain not only vaginal secretions but also semen or even saliva left by the suspect. No cross-reaction was found from a mixture of blood and other body fluids in this study. Besides, no positive result was found within blood samples from other animal species. Nevertheless, more cross-reaction tests for the mixtures with other animal species not included in this study should be evaluated before practical usage in forensic investigation and more attention should be paid to representing the result from an alleged mixture of body fluids.

In this study, it was proved that LAMP showed high potential for providing the forensic scientists an ideal tool in blood identification for its simplicity, efficiency, specificity, and sensitivity. RT-LAMP takes only less than 1 hour to complete the whole reaction. Traditional two-step procedures including reverse transcription and PCR comparatively cost more expense, time, and labour for the whole experiment and raise a higher risk of contamination. Besides, traditional PCR-based methods require a heavy and expensive thermal cycler and equipment for reaction, making it difficult to perform these methods at the crime scene. In this study, fluorescent detection of RT-LAMP was also demonstrated for forensic experts to easily monitor the presence of fluorescence remotely from the laboratory.

This is the first application of real-time RT-LAMP on detection of body fluid-specific RNA. The results have demonstrated the potential of applying this method on forensic biology. As blood is the most common biological evidence found at the crime scene, identification of the blood was used as a proof-of-concept model in this study. However, the expected limitations for expanding RT-LAMP technique to body fluids other than blood will be the insufficient RNA quantity and quality from the trace or degraded samples. Therefore, more sensitive conditions and strategies of RT-LAMP need to be developed to overcome the obstacles.

To sum up, we demonstrated a novel application of real-time RT-LAMP in this study to determine the presence of a body fluid, using blood as a model. The results showed that the method is reproducible, specific, sensitive and time-saving. Another advantage of RT-LAMP is that the test could be performed using one piece of laboratory equipment, instead of a dedicated machine. It was also shown that detecting fluorescence with the presence of calcein from the RT-LAMP products could be performed simply and is applicable to testing remotely from the laboratory such as at a crime scene. The assay was applied successfully to the putative identification of blood from 21 non-probative forensic samples, including two old bloodstains which had been stored in envelopes for 12 years. This demonstrated that LAMP is capable of identifying old body fluid stains.

Chapter 4 Identification of saliva by real-time RT-LAMP

4.1 Introduction

A model for blood identification by LAMP was established and reported in the previous chapter, the results showed that LAMP can be a novel method for body fluid identification. As blood is a body fluid found not infrequently at crime scenes, many studies have focused on blood identification and there are several methods and commercial kits available for this purpose. Besides blood, saliva is another body fluid often left at the crime scene but may be missed because there is no distinctive natural colour. Several methods for identification of saliva have been proposed in literature (see Section 1.1.3), but most of them like other presumptive tests lack sensitivity and specificity. In this chapter, the results of the alternative method of applying real-time RT-LAMP to saliva identification by utilizing saliva-specific mRNA markers were reported.

4.1.1 Saliva

The principal component of human saliva is water (98%). The other 2% is composed of electrolytes, mucus, antibacterial substances, enzymes, and epithelial cells. Saliva helps to moisten the food, making it easier to swallow. One of the enzymes in saliva is amylase which breaks down starch. Saliva is invariably left on the containers of food or drinks. Thus, saliva evidence can be usually found on the edge of a cup or the top of a bottle.

Saliva production is rather complicated. Around 70% of salvia is secreted by the submandibular salivary glands, about 25% by the parotid glands, and 5% by the

sublingual salivary glands (Figure 4-1) [289]. These glands are distributed in the entire mouth except the gums and the front palate. The salivary glands are composed of acinic cells and tunnel cells. Some glands secrete saliva and some produce mucus. In addition, there are also mixed glands that can produce both. The acinic cells of the parotid gland generate extensive secretion and this gland synthesizes most of the α-amylase. The calcium it generates is less than that generated by the submandibular glands. The primary ingredient of saliva is mucin, which is mainly secreted by the submandibular and sublingual glands. Other ingredients, such as proline-rich glycoprotein, are synthesized by the parotid glands [290]. The minor salivary gland is basically a mucus gland that mainly generates mucus saliva.

Approximately 500-700 ml of saliva is produced every day. The rate of saliva secretion can rise to 1 ml per minute under certain stimulation. Under normal situation without external or medical stimulation, a small amount of saliva is continuously produced in the mouth, which helps to keep moisture of mouth.

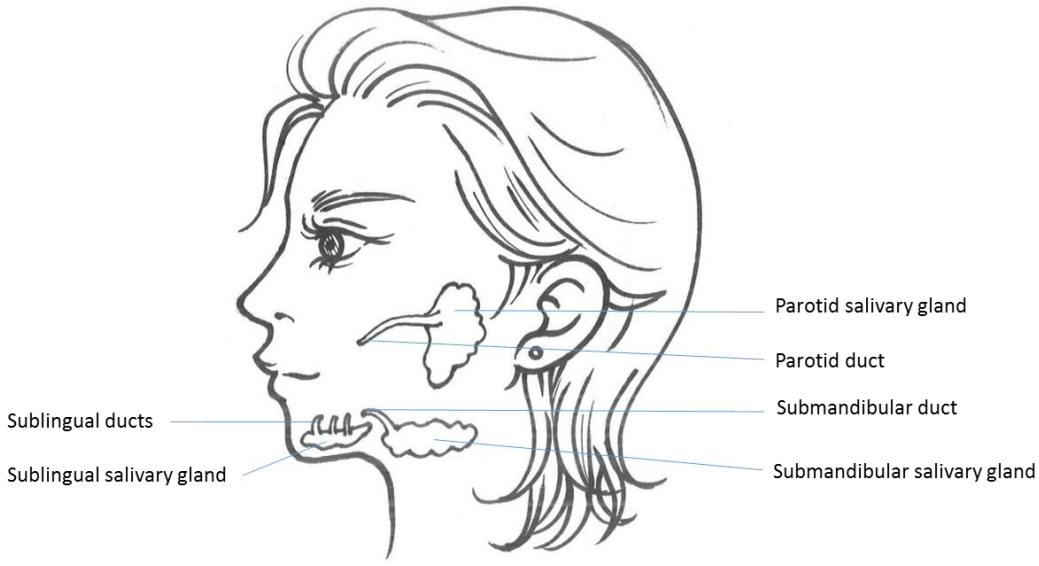


Figure 4-1 An illustration of human salivary glands

The amount of saliva in oral cavity is normally about 1.1 ml and reaches its peak during the meal time, usually around noon time, and then decreases significantly during the sleep at night. In addition to 98% of water, saliva also contains many different electrolytes such as sodium, potassium, calcium, magnesium, chloride, bicarbonate, phosphate and so on. Saliva is sterile when it is initially secreted, but bacteria may be present depending on oral health.

The saliva proteins include enzymes, immunoglobulins, antibacterial factors and glycoproteins, all crucially related to the sanitary condition of oral cavity. There are also some glucose and nitrous substances, such as urea and nitrogen. The composition and function of saliva is listed in Table 4-1 [291, 292].

Amylase is considered as a suitable target for identification of saliva and is currently used by forensic analysts as a presumptive test. It is an enzyme that breaks down carbohydrates by breaking the glycosidic bonds between monomers. There are 3 amylase forms, including α-amylase, β-amylase, and γ-amylase. In humans, α-amylase is present in both salivary and pancreatic secretions and is produced by two different loci on chromosome 1, *AMY1* (salivary locus) and *AMY2* (pancreatic locus), respectively [293-296]. Salivary amylase is abundant in human saliva and there is also a small amount of salivary amylase in breast milk and sweat which can produce false positive results when aiming to identify saliva with salivary amylase. Pancreatic amylase is found in urine, semen, faeces and vaginal secretions [297]. Pancreatic amylase, secreted by the pancreas, completes digestion of carbohydrate in small intestine. Thus, pancreatic amylase may also result in false positive reactions using detection of this enzyme as a presumptive test for saliva. The range of amylase

concentration in forensically relevant body fluids [298] is shown in Table 4-2. Accordingly, urine is the second highest amylase-containing body fluid (1/1000X). Besides, traces of faeces may also contain amylase activity, leading to false positives.

Table 4-1 Composition and function of saliva

Functions	Components
Lubrication	Mucin, proline-rich glycoproteins, water
Antimicrobial action	Lysozyme, lactoferrin, lactoperoxides, mucins, cystins, histatins, immunoglobulins, proline-rich glycoproteins, IgA
Maintaining mucosa integrity	Mucins, electrolytes, water
Cleansing	Water
Buffer capacity and remineralisation	Bicarbonate, phosphate, calcium, statherin, proline-rich anionic proteins, fluoride
Preparing food for swallowing	Water, mucins
Digestion	Amylase, lipase, ribonucleases, proteases, water, mucins
Taste	Water, gustin
Phonation	Water, mucin

Adapted from Puy, Carmen Llena. "The role of saliva in maintaining oral health and as an aid to diagnosis." Med Oral Patol Oral Cir Bucal 11 (2006): E449-55.

Table 4-2 The range of amylase concentration in forensically relevant body fluids.

Body fluid	Amylase activity (IU/L)
Saliva	263000 to 376000
Urine	263 to 940
Blood	110
Semen	35
Nasal secretion	Undetectable levels
Sweat	Undetectable levels

Adapted from Whitehead, P. and A.E. Kipps, A test paper for detecting saliva stains. Journal of the Forensic Science Society, 1975. 15(1): p. 39-42

Numerous bacteria exist in the oral cavity, so some forensic scientists tried to identify saliva by detecting oral bacteria. Nakanishi *et al.* indicated that streptococci can be a promising new marker for the forensic identification of saliva and demonstrated a method for the identification based on PCR [299]. Chio *et al.* represented a method to identify saliva using integrated analysis of DNA methylation and specific microbial DNA [181]. Recently, mRNA markers have been identified in a number of studies for the forensically relevant body fluids, including saliva, based on functional differences of the cells (see Section 1.3).

4.1.2 Specific mRNA markers for saliva

Several candidate markers have been evaluated for their saliva-specific expression (listed as Table 1-3 in Chapter 1). Some studies have confirmed that *STATH*

and *HTN3* mRNA markers are present in saliva but absent in blood, semen, saliva, vaginal secretion and menstrual blood [128]. Besides, several stable saliva-specific mRNA markers have been revealed for their potential of body fluid identification [127, 144, 148, 262]. Among these saliva-specific markers, *SPRR1A*, *SPRR2A*, *KRT4*, *KRT6A* and *KRT13* mRNA were screened by bio-chip and also confirmed using real-time PCR by Zubakov [127]. The result revealed that the *SPRR1A* marker showed no detectable expression in semen after 50 RT-PCR cycles. As for *KRT4*, *KRT6A* and *KRT13* mRNA, some product was detected in semen samples but was at a much lower level than in saliva. A multiplex system was developed for body fluid identification. *KRT4*, *KRT13* and *SPRR2A* mRNA were included as mucus-specific instead of saliva-specific. *KRT4* and *SPRR2A* mRNA were found not only in saliva samples but also in vaginal secretion and mucosa. *KRT13* mRNA was found only in saliva, vaginal secretion and mucosa and at a much lower level in semen [148, 262].

In addition to the scientific literature, the BioGPS database [300] was also used for marker selection of saliva in this study. BioGPS database was established by Genomics Institute of the Novartis Research Foundation (GNF) [301]. This institute also responds to enquiries and organizes gene annotation resources. Based on the references and inquiry to BioGPS, *STATH*, *HTN3*, *SPRR1A*, *SPRR2A*, *KRT4*, *KRT6A* and *KRT13* mRNA were selected for future saliva identification (Appendix 5). All the selected markers are discussed below.

4.1.2.1 *STATH*

Statherin is a salivary protein encoded by *STATH* gene and helps to inhibit the nucleation and growth of hydroxyapatite crystals in the oral cavity [302, 303]. It plays

an important role in maintaining the mineralization of enamel surface and promoting antimicrobial function. Based on several studies, the *STATH* mRNA has been shown as a specific marker for saliva identification [148, 304]. In addition, according to BioGPS database, it is highly expressed in salivary glands and trachea while it has limited expression in thyroid, prostate and thalamus (Appendix 5). Therefore, *STATH* mRNA was evaluated as a candidate marker for saliva identification in this study.

4.1.2.2 *HTN3*

Histatins are a group of histidine-rich proteins with low molecular weight, mainly secreted by parotid glands, submandibular glands, and sublingual glands of humans or higher primates. They are antimicrobial and antifungal proteins which play a role in wound-closure [305-307]. Among the family members of histatins, histatin 2 is a proteolytic product form of histatin 1 while all the other histatins are hydrolysed from histatin 3 [308, 309]. Histatin 3 is encoded by *HTN3* genes. According to BioGPS database, *HTN3* genes have salient expression in salivary glands and very low expression in the rest of the tissues except a relatively small amount in the thyroid, prostate and the hypothalamus (Appendix 5).

4.1.2.3 *KRT4*, *KRT6A* and *KRT13*

Keratin is a rigid fibrous protein which is the key structural material of the outer layer of skin cells and protects epithelial cells from damage. It is also the major structural component in hair and nails [310]. During progressive differentiation of stratified epithelia, the epidermal cells synthesize a sequence of different keratins as they mature [311]. The primary keratins expressed in the basement membrane are

keratin 5 and keratin 14 while keratin 6, 16, 4, and 13 can be found in the mucosal epithelia [312]. Keratin 4 and 13 proteins are translated from *KRT4* and *KRT13* mRNA respectively while keratin 6A is encoded by *KRT6A* genes, an isomer of keratin 6, and found in the palms and soles of the epidermis, epidermal cells of the fingernail bed, filamentous papillae on the tongue, oral mucosa and epithelial lining of oesophagus and in the hair follicle.

According to BioGPS database, *KRT4* gene is expressed in the tonsils and tongue, but only a low level or no expression is found in the tissues of other organs (Appendix 5). *KRT13* genes are mostly expressed in the tongue and tonsils, and only a low level of *KRT13* gene is expressed in other tissues (Appendix 5). *KRT6A* genes are mainly expressed in the tongue and bronchial epithelial cells, with lower expression in tonsils and placenta, and a low level or no expression is found in the tissues of other organs (Appendix 5). However, as mentioned in the previous section, the mucosa markers, including *KRT4* and *KRT13* mRNA, are detected not only in saliva but also in vaginal mucosa and menstrual secretion [148]. *KRT13* mRNA is found in saliva, vaginal secretion and mucosa and at a relatively low level in semen [262].

4.1.2.4 *SPRR1A* and *SPRR2A*

A group of small proline-rich proteins are encoded by the *SPRR* gene family. When human epidermal keratinocyte proliferation undergoes differentiation and proliferation, a large number of such proteins are activated. The *SPRR* family consist of three subclasses of genes including SPRR1, SPRR2, and SPRR3. Two *SPRR1* genes (*SPRR1A* and *SPRR1B*), six *SPRR2* genes (*SPRR2A, B, D, E, F,* and *G*), one *SPRR3* gene, and one *SPRR4* gene are found on human chromosome 1 region q22-q23 [313, 314]. The *SPRR* genes are expressed in squamous tissues of the skin, scalp, and vaginal

epithelia. These genes are also expressed in most of the epithelia lining of the digestive tract [315]. *SPRR3* protein is largely expressed in the oral and oesophageal epithelial cells but not in epidermal cells. On the contrary, *SPRR2* protein is primarily expressed in the epidermal cells while expression of *SPRR1* protein is confined to appendageal regions [316].

SPRR proteins play an important role in would healing and also involve in skin cornification [317]. The expression of *SPRR* genes is regulated by UV irradiation or the treatment with the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) [318]. According to BioGPS database, *SPRR1A* gene is expressed primarily in tongue, next in tonsil, while rarely expressed in other tissues and organs (Appendix 5). As for *SPRR2A* gene, its specific expression has not yet been registered in BioGPS database though it has been detected in saliva, vaginal mucosa, menstrual secretion and skin samplings [148].

4.2 Materials and methods

The flow chart of the experiment is shown as Figure 4-2.

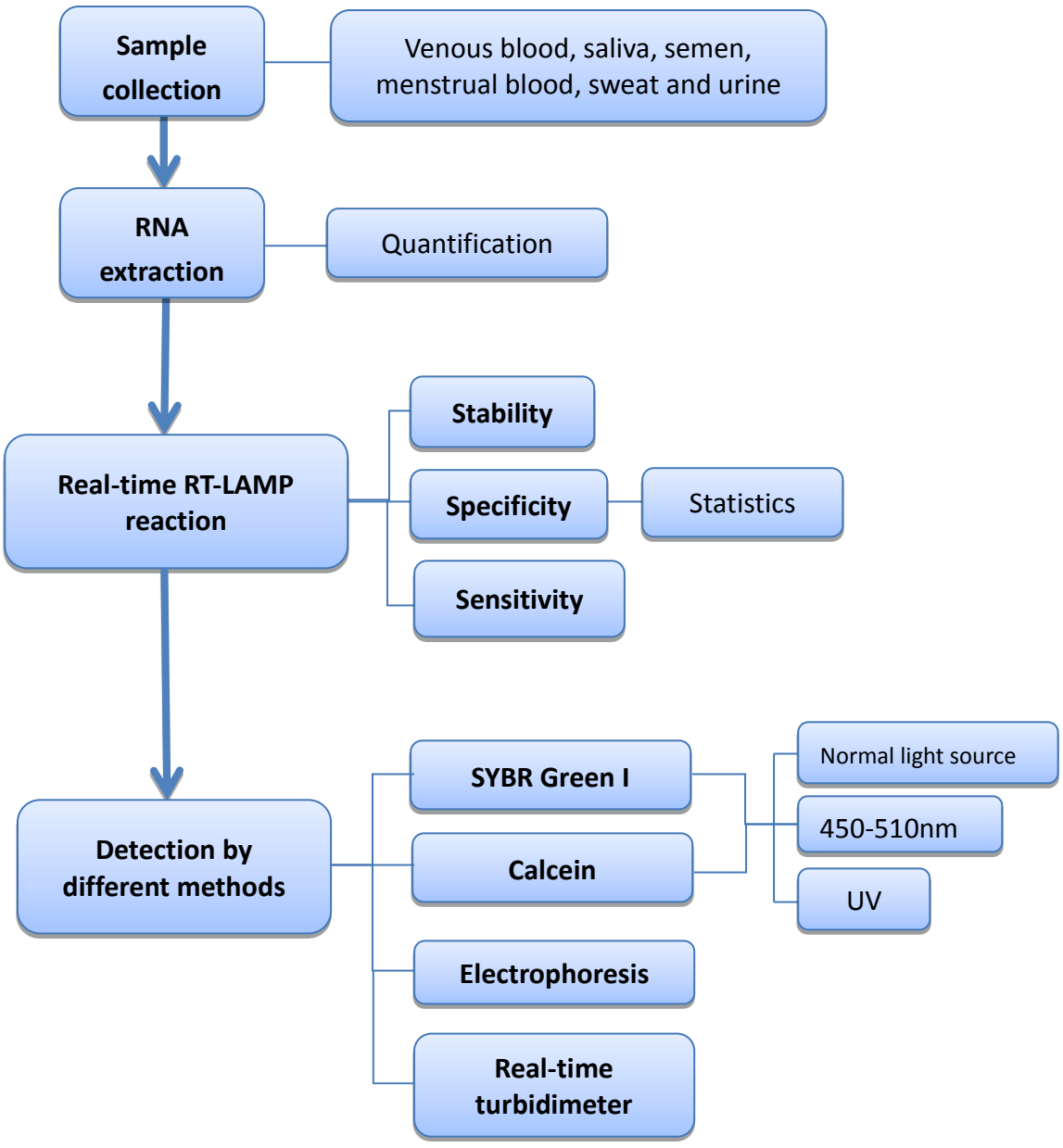


Figure 4-2 Flowchart of this study

4.2.1 Sample collection

Different body fluids (including blood, menstrual blood, saliva, semen, sweat, urine, and vaginal secretion) were tested in this study. The samples for each body fluid were collected from 12-17 consenting volunteers aged from 20 to 40 years old using procedures approved by Institutional Review Board (IRB) of Central Police University in Taiwan and National Research Ethics Service (REC reference number: 10/H0808/94, Appendix 1).

Whole blood samples (4ml) were collected by a qualified phlebotomist in a clean, private first aid room. The menstrual blood was collected from healthy volunteers. In order to get best results, menstrual blood was collected on the day of the volunteer's heaviest menstrual flow. The volunteers inserted tampon themselves to collect samples. The menstrual blood was then collected by squeezing the tampons. Freshly ejaculated semen samples were collected by the volunteers using a sterile plastic container. Saliva samples (2ml) were collected in a sterile plastic container after volunteers have rinsed their mouths before taking the sample. For sweat samples, volunteers were asked to exercise for approximately 20 minutes before collecting approximately 5ml of their sweat in sterile containers. Urine samples (at least 50ml) were collected from volunteers with tubes for urine collection. Vaginal secretion samples were collected by volunteers with swabs and stored in a freezer at -20°C before RNA extraction. All the equipment used in this procedure was sterile and disposable to minimize the risk of infection and contamination.

4.2.2 Extraction and quantification of RNA

Total RNA was extracted from each body fluid with RNeasy® Mini Kit (Qiagen® Ltd, UK) according to the protocol for purification from animal tissues (described in Section 2.1.1) with some modifications depending on the body fluids (described in the previous chapter). Total RNA (2µl) was then used for the quantification using a Nanodrop spectrophotometer (described in Section 2.1.2).

4.2.3 Marker selection

18S rRNA marker was selected as the internal control as mentioned in the previous chapter. Seven saliva specific markers (Table 4-3) were adopted in this study based on their specificity for saliva (see Section 4.1.2).

Table 4-3 Specific markers selected for saliva identification in this study

Gene	Location	Target	Function	Reference
18S rRNA	22p12	Housekeeping	Human 18S ribosomal RNA	[148, 319]
HTN3	4q13	Saliva	Histatin 3	[128, 320]
STATH	4q13.3	Saliva	Statherin	[128]
SPRR1A	1q21-q22	Saliva	Small proline-rich protein 1A	[321]
SPRR2A	1q21-q22	Saliva	Small proline-rich protein 2A	[321]
KRT4	12q13.13	Saliva	Keratin 4	[321]
KRT13	17q21.2	Saliva	Keratin 13	[321]
KRT6A	12q13.13	Saliva	Keratin 6A	[321]

4.2.4 Design of LAMP primers

For each marker (except *18S rRNA*), at least one of the primers was designed to span intron/exon junctions to prevent amplification from any contaminating DNA. Primers were designed by PrimerExplorer V3 software on internet website (http://primerexplorer.jp/elamp4.0.0/index.html, Eiken Chemical Co). The sequences of all the primers are shown in Table 4-4. The locations of 6 specific sites for *18S rRNA*, *HTN3*, and *KRT4* are shown in Appendix 6.

Table 4-4 Sequence of primer sets designed for *18S rRNA* and saliva specific markers

Marker	Primer	Sequence (5'-3')
18S rRNA[#]	F3	ATTGACGGAAGGGCACCA
	B3	TGCCAGAGTCTCGTTCGTTA
	FIP	CAATCCTGTCCGTGTCCGGGAGCCTGCGGCTTAATTTGAC
	BIP	AGCTCTTTCTCGATTCCGTGGGAGACAAATCGCTCCACCAAC
HTN3	F3	GTTTTTGCTTTAATCTTGGCTC
	B3	TGCGGTATGACAAATGAGAA
	FIP*	GATGTGAATGATGCTTTTCATGGAACTTTCCATGACTGGAGCT
	BIP	TTGATATCTTCAGTAATCACGGGGCGAGTCCAAAGCGAATTTGC
KRT4	F3	GCGTGGAGGACTTCAAGACT
	B3	GTCGCTGACATGGGTCTG
	FIP	AGGCAGCATCCACGTCCTTCTTAGGAGATCAACAAACGCACA
	BIP*	TGGAGTTGGAGGCCAAGGTGGTGGGACAGCTCCGCATC
KRT6A	F3	CCCTCAACAACAAGTTTGCC
	B3	CCTCTGAGCTCTGAGTCCAG
	FIP*	TGCCCTGCTCCTGCAGCAGACAAGGTGCGGTTCCTGG
	BIP	GGAGCCGTTGTTCGAGCAGTCCCCGTTCCCCGACAAT
KRT13	F3	AGCCCCTACTACAAGACCAT
	B3	GCTCTCGATCTGCATCTCCA
	FIP*	AGCCAGCCTGGCATTGTCAATGGGACAAGATCCTGACCGC
	BIP	TATGAGAATGAGCTGGCCCTGCAGCTCATCCAGCACCCG
STATH	F3	TTCTGTAGTCTCATCTTGAGT
	B3	TGTGGGTATAGTGGTTGTTC
	FIP*	GAAACCATGAGAGCCAAGATGAAAGAGAACCCAGCCAACT
	BIP	ATGATTGGAGCTGATTCATCTGAAGGAACTGGCTGATAAGGGC
SPRR2A	F3	TGGTACCTGAGCACTGATCT
	B3	GGCTGTGGACACTTTGGT
	FIP*	TGCTTGCACTGCTGCTGTTGATGCCTTGGAGAACCTGATCCT
	BIP	CCAGCCACCTCCTGTGTGCAGGGCTCAGGGCACTTC

* The primer was designed to include a sequence of the intron-exon junction.

The primers for *18S rRNA* in this chapter is the same as in Chapter 3.

4.2.5 Real-time RT-LAMP reaction

RT-LAMP was conducted with Loopamp® RNA Amplification Kit (Eiken Chemical Co. Ltd.; Tochigi, Japan) following the progress described in Section 2.4.2.2. The total volume for the entire reaction was 25µl which consisted of 40µM of each inner primer FIP and BIP, 5µM of each outer primer F3 and B3, 12.5µl reaction mix, 1U enzyme and 5µl RNA template (extracted from 50µl body fluids). For real-time LAMP detection, a reaction was performed using the real-time turbidimeter LA-500 (Eiken Chemical Co. Ltd., Tochigi, Japan). The thermal program was set at 65°C for 60 minutes and then 80°C for 5 minutes. For each marker, preliminary tests were performed to screen the specificity of the primer sets. Further stability and reproducibility tests with different body fluids were conducted in triplicate. Negative controls that contained everything but RNA were also tested in the reactions.

Beside real-time LAMP detection by the real-time turbidimeter, the LAMP products were also detected by electrophoresis and fluorescence. For electrophoresis, an aliquot (1.5µl) was added to loading buffer and electrophoresis was performed using 2 % agarose gel and SYBR® Green in 1X TBE buffer at 120V and 400mA for 40 minutes. The gel image was then photographed under UV transillumination (Syngene® GBox, Maryland, USA) using Genesnap® from Syngene® image acquisition software (Vision-Capt version 14.2). For fluorescence detection, both calcein and SYBR® Green I were used in this study (see Section 2.4.3). SYBR® Green I was added to the reagents after reaction. The products were observed with the naked eye under normal lighting or under 450-510nm aquamarine blue light with a 600nm filter or 365nm UV with a 570nm filter. The images were captured using a digital camera. Unlike SYBR® Green I that was added after LAMP reaction, calcein was added to the reagents before performing LAMP reaction. The products could be observed with the naked eye under

normal lighting or under 450-510nm aquamarine blue light with an orange filter or 365nm UV with a yellow filtered goggle. The images were, once again, captured by a camera.

4.2.6 Statistics for evaluating the markers

Ideally, a perfect diagnostic method should reflect the true result without any false positive or false negative. Unfortunately, most methods have their own limitation and it is difficult to get rid of false results in normal condition. Hence, it is important to evaluate the performance of a testing method. Results of the validation for a test prediction rule can be described using a number of statistics. The most commonly used methods are accuracy, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and likelihood ratio (LR).

When a test is applied for an examination, a true positive represents that a sick person is correctly identified as sick. On the contrary, when a healthy person is wrongly diagnosed as sick, it is defined as false positive (also known as type I error, which is usually the result that we want to avoid). On the other hand, a true negative represents that a healthy person is correctly identified as healthy while a false negative (type II error) represents that a sick person is wrongly identified as healthy. The four outcomes can be formulated as a 2×2 contingency table (Table 4-5) and used for further statistical calculation.

Table 4-5 The contingency table for a binary classification that visualises the performance of a test

		True Condition (gold standard)		
		Condition Positive (CP)	Condition Negative (CN)	
Test Outcome	Test Outcome Positive (TOP)	True Positive (TP)	False Positive (FP, Type I error)	Positive Predictive Value (PPV) = TP/TOP
	Test Outcome Negative (TON)	False Negative (FN, Type II error)	True Negative (TN)	Negative Predictive Value (NPV) = TN/TON
		Sensitivity= TP/CP	Specificity= TN/CN	

Accuracy (ACC) = (TP+TN) / (CP+CN)
False positive rate = FP / (FP+TN) = 1-specificity
False negative rate = FN / (TP+FN) = 1-sensitivity
Positive Likelihood Ratio (LR+) = Sensitivity / (1-Specificity)
Negative Likelihood Ratio (LR-) = (1-Sensitivity) / Specificity

Sensitivity (also known as the true positive rate) refers to the ability of a method to identify a true positive condition correctly. On the contrary, specificity (also known as the true negative rate) indicates the ability of a test to rule out a negative condition correctly. The limitation of both sensitivity and specificity is that they are based on the assumption that the actual condition is known. However, analysts use a test because they actually do not know the real condition and they need the result of the test to guide us for the identification of body fluid.

Compared with both sensitivity and specificity, PPV and NPV are more useful in the real world. In medical diagnosis, PPV is the probability that subjects with a positive screening test truly have the disease. In other words, it is the proportion of patients with a positive result who actually have a disease. On the contrary, NPV

represents the proportion of patients with a negative result who are actually free of disease. Nevertheless, predictive values are not stable as they are affected by the prevalence of the disease. Ebell made a comparison among some statistical methods [322] for the measurement of the performance of a test and summarized the characteristics of these statistical methods (Table 4-6). Likelihood ratio is considered as the best tool among these methods [322]. It is defined as how much more likely it is that a patient who tests positive has the disease compared with one who tests negative.

Table 4-6 The characteristics of the sensitivity, specificity, predictive value, and likelihood ratio

Test	Clinical meaning	Stability with changing prevalence	Can use multiple levels of a test result
Sensitivity or specificity	No	Yes	No
Predictive value	Yes	No	No
Likelihood ratio	Yes	Yes	Yes

Adapted from Ebell, M.H., Evidence-based diagnosis: a handbook of clinical prediction rules. Vol. 1. 2001: Springer Science & Business Media

The sensitivity, specificity, predictive values, and likelihood ratio for statistical analysis were applied in this study to determine the reliability of the markers for saliva identification. Besides, accuracy is also used in this study to evaluate the markers. The formula for calculating these measures are listed in Table 4-5.

4.3 Result

4.3.1 Preliminary tests of the markers

Before further analysis, each marker was tested with different body fluids (including blood, saliva, semen, menstrual blood, sweat, and urine) and negative control samples in triplicate to screen the markers.

Among these markers, no primer set was found for the *SPRR1A* marker with PrimerExplorer software. In designing LAMP primers, there are several key factors for designing of the primers, including melting temperature, stability at the end of the primers, GC content of primers, secondary structure, and distance between primers (see Section 2.4.1.2). Based on the estimation by this PrimerExplorer, there was no proper primer site for the *SPRR1A* marker, so it was excluded in the preliminary test in this study. The result of the preliminary test is listed in Table 4-7. Among the results, no amplification was found in negative control samples and saliva samples for *KRT6A* and *STATH* markers. Therefore, *KRT6A* and *STATH* markers were also excluded in this study.

Four potential saliva-specific markers, *HTN3*, *KRT4*, *KRT13*, and *SPRR2A*, were further evaluated. The *HTN3* marker was only detected in saliva samples (Tt = 37.2±2.9) and no amplification was found in the other tested body fluids or negative samples by real-time RT-LAMP reaction. Based on the consistent reproducibility in the preliminary study, *HTN3* marker was chosen for further identification of saliva.

LAMP products with the *KRT4* marker were detected in both saliva (Tt = 38.1±3.1) and menstrual blood (Tt =43.8±1.6) samples. This result was consistent with the study of Lindenbergh *et al*. which reported that *KRT4* could be found in saliva, vaginal mucosa, and menstrual secretion [148]. Although the *KRT4* marker is not only specific for saliva, it still can be useful for classifying the body fluids. Besides, it can

assist in confirming a positive result of saliva with the other saliva-specific markers by LAMP or with the other presumptive identification methods. Thus, the KRT4 marker was also adopted for further evaluation.

Regarding the *KRT13* marker, no amplification was found in negative control samples. However, not all of the three saliva samples were tested positive in the preliminary test (two positives and one negative). Besides, one semen sample was tested positive, which is also mentioned in the study of Lindenbergh *et al.* which reported that *KRT13* mRNA was found in saliva, vaginal secretion and mucosa and at relatively low levels in semen [148]. As a result, this marker may not be good enough for the identification of saliva owing to low sensitivity and specificity (0.68 and 0.83 respectively, calculated as Table 4-8). Thus, the *KRT13* marker was not used for further analysis.

As for the *SPRR2A* marker, no amplification was found in negative samples. However, LAMP products with this marker were detected in all body fluids except the blood samples and one of the urine samples. The result consisted with the description in Section 4.1.2.4. Therefore, the *SPRR2A* marker was not further included for the following analysis.

Based on the results of the preliminary test shown above, two markers (*HTN3* and *KRT4*) were chosen for further study. Besides, an additional internal control marker (*18S rRNA*) which had been tested earlier in the previous chapter was also used in this study.

Table 4-7 Analysis of the expressions for 6 markers performed in triplicate using one sample from each body fluid using real-time RT-LAMP

Marker	Test no.	Tt(min)						
		Blood	Saliva	Semen	Menstrual blood	Sweat	Urine	NC
HTN3	1	-	40.0	-	-	-	-	-
	2	-	34.2	-	-	-	-	-
	3	-	37.5	-	-	-	-	-
	Avg±S.D.	-	37.2±2.9	-	-	-	-	-
KRT4	1	-	35.8	-	42.0	-	-	-
	2	-	41.6	-	44.3	-	-	-
	3	-	36.8	-	45.1	-	-	-
	Avg±S.D.	-	38.1±3.1	-	43.8±1.6	-	-	-
KRT6A	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	Avg±S.D.	-	-	-	-	-	-	-
KRT13	1	-	-	-	-	-	-	-
	2	-	32.6	42.8	44.8	-	-	-
	3	-	55.0	-	54.2	-	-	-
	Avg±S.D.	-	-	-	-	-	-	-
SPRR2A	1	-	37.5	54.9	37.3	44.6	-	-
	2	-	37.6	46.0	36.7	47.1	55.6	-
	3	-	38.8	56.0	39.0	52.3	42.2	-
	Avg±S.D.		38.0±0.7	52.3	37.3±1.2	48.0±3.9	-	
STATH	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	Avg±S.D.	-	-	-	-	-	-	-

NC: Negative control.

- : No amplification was detected in 60 minutes.

S.D.: The average value with a standard deviation.

Table 4-8 The calculation of the sensitivity and specificity for the *KRT13* marker as a saliva-specific maker in the preliminary test

	Saliva samples	Non-saliva samples
Positive result	2	3
Negative result	1	15

Sensitivity (true positive rate) = true positives / (true positives + false negatives) = 2/(2+1) = 0.67
Specificity (true negative rate) = true negatives / (true negatives + false positives) = 15/(15+3) = 0.83

4.3.2 Specificity test of the markers

All the three markers (*HTN3*, *KRT4* and *18S rRNA*) were evaluated with specificity test respectively. The *18S rRNA* marker had been used as the internal control as reported in the previous chapter. In this study, more samples (at least 12 samples for each body fluid) were tested by real-time RT-LAMP and the result is shown in Appendix 7. The threshold time ranged from 30.4 to 52.0 minutes for blood samples, 30.7 to 48.2 minutes for saliva samples, 26.3 to 59.3 minutes for semen samples, 30.7 to 44.3 minutes for menstrual blood samples, 34.1 to 57.4 minutes for sweat samples, 40.9 to 55.9 minutes for urine samples, and 36.7 to 53.6 minutes for vaginal secretion samples respectively. The average and standard deviation of the threshold time of RT-LAMP reaction for each body fluid are shown in Figure 4-3.

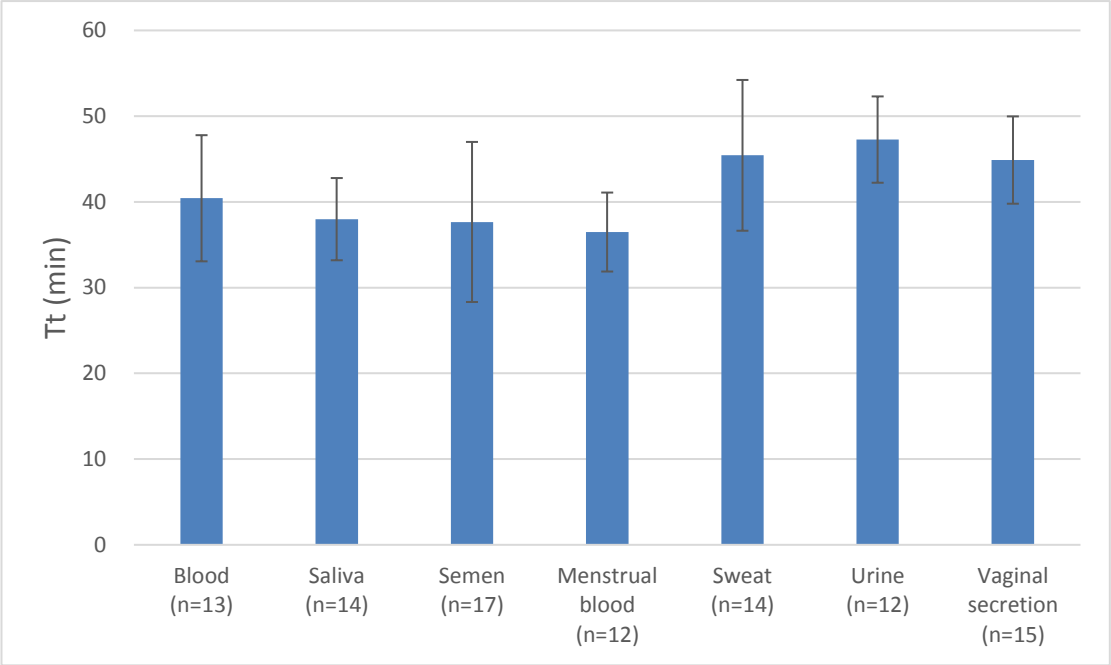


Figure 4-3 The average and standard deviation of the threshold time of RT-LAMP reaction for different body fluids with the *18S rRNA* marker

The results showed that all the body fluids could be detected by *18S rRNA* marker as expected. No LAMP product was detected in negative controls. Thus, *18S rRNA* marker is capable of being used as the internal control for body fluid identification. The *18S rRNA* is essential for the ribosome and makes up about 20% of total RNA [323]. Its proportion among total RNA remains stable. Thus, the deviation of the threshold time from each sample should keep in a low level. However, comparing the threshold time of different individuals and body fluids, it varied a lot in some samples. Several factors might lead to the consequence. First of all, RNA degradation is a prevalent activity [324]. Although all of the total RNA were extracted from the body fluids as soon as they were collected, the rate of RNA degradation was not expected to be equal for each sample. Besides, the extracted total RNA was quantified by UV spectrophotometer which measured the absorbance of 260 nm and 280 nm wavelength to estimate the concentration of total RNA. Although

quantification by UV spectrophotometer is convenient, it is not accurate [325]. The inaccurate estimation of the quantification would cause variation in the amount of total RNA added in the reaction mixture and further lead to variation of the threshold time. Moreover, LAMP reaction is so sensitive that even a slight difference in concentration of RNA, primers, or reagents may result in different efficiency of amplification. Thus, human errors, such as pipetting errors, may also cause this kind of result.

Concerning the performance of the *HTN3* marker, LAMP product was found in all saliva samples. The threshold time ranged from 34.5 to 46.8 minutes. The average was 40.5 minutes with a standard deviation of 3.6 (Figure 4-4). Beside saliva samples, only one sweat sample was detected as positive in 40 minutes (Appendix 7). The same sweat sample was confirmed as positive again after a repeated experiment. An investigation into the progress of sample collection with the volunteer revealed that the donor put the collection tube around his lips when collecting the sweat. Hence, saliva might be mixed with the sweat during this collection, resulting in the irregular result of this sample. This sample was ignored in the following analysis. The final result of the *HTN3* marker was consistent with the result in previous studies [156, 306, 320], showing that the *HTN3* marker is saliva-specific.

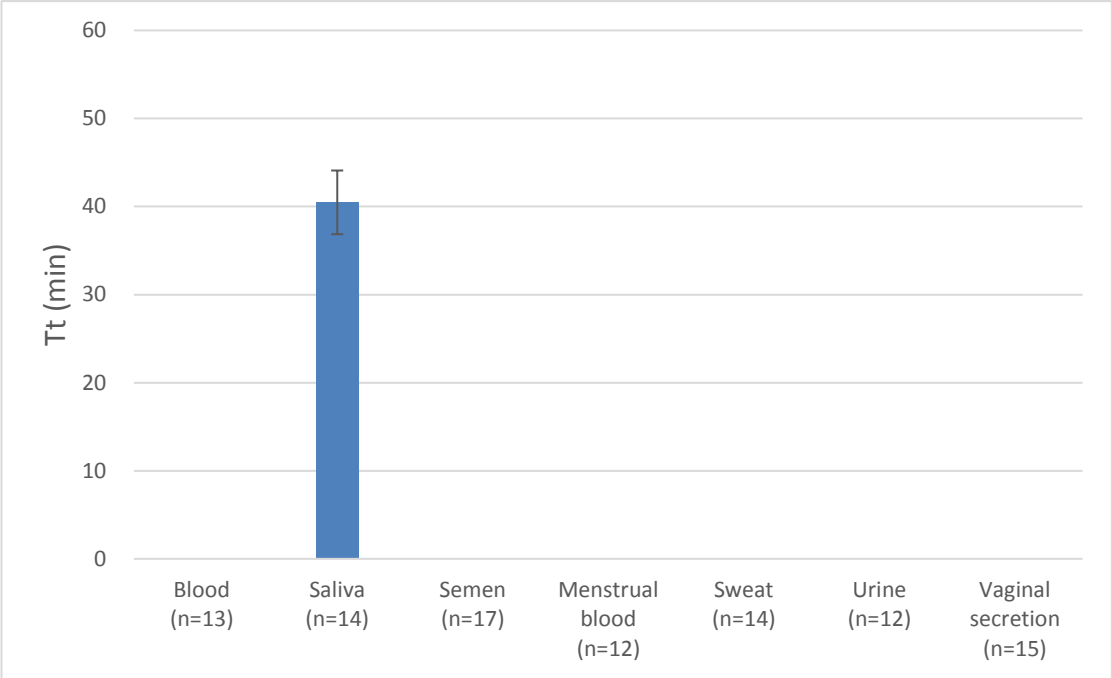


Figure 4-4 The average and standard deviation of the threshold time of RT-LAMP reaction for different body fluids in *HTN3* marker

As for the *KRT4* marker, LAMP product could be detected within 60 minutes for all saliva samples. The threshold time ranged from 34.4 to 49.3 minutes with an average of 39.4 minutes and a standard deviation of 3.8. No LAMP product was found within 60 minutes for blood and sweat samples. However, some menstrual blood (7/14), urine (8/14), and vaginal secretion (7/15) samples and one semen sample were tested with positive result (Appendix 7). This indicated that *KRT4* might lead to false positive. To further evaluate this marker, statistical analysis using binary classification of sensitivity and specificity was performed in the next section.

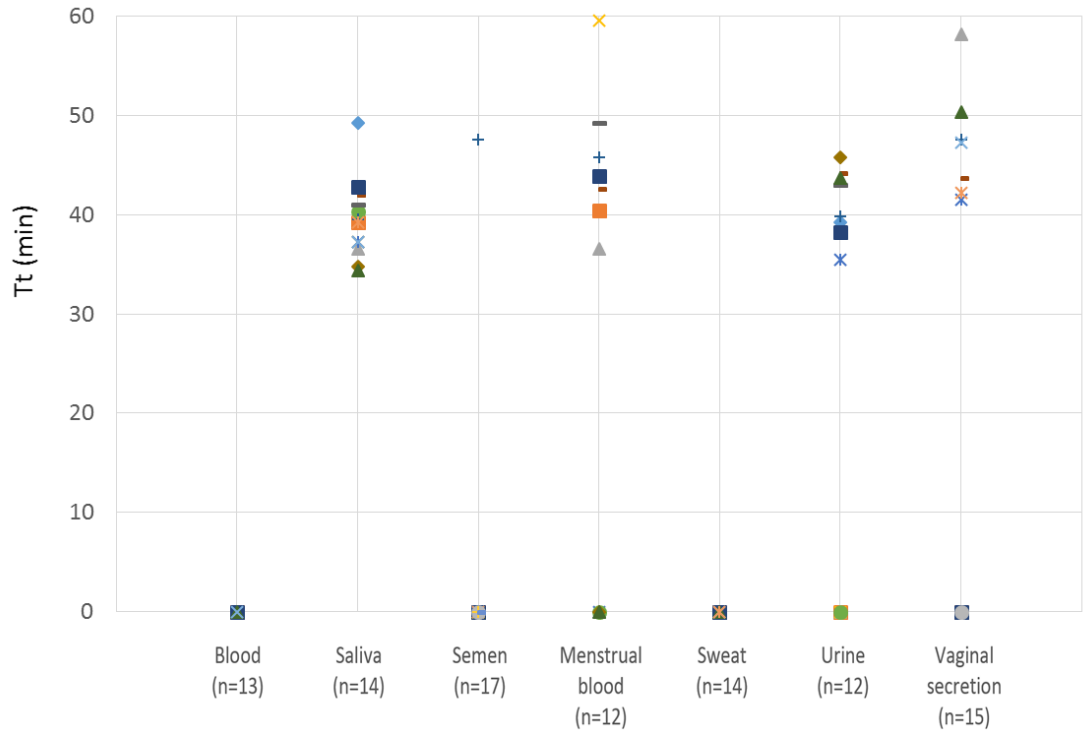


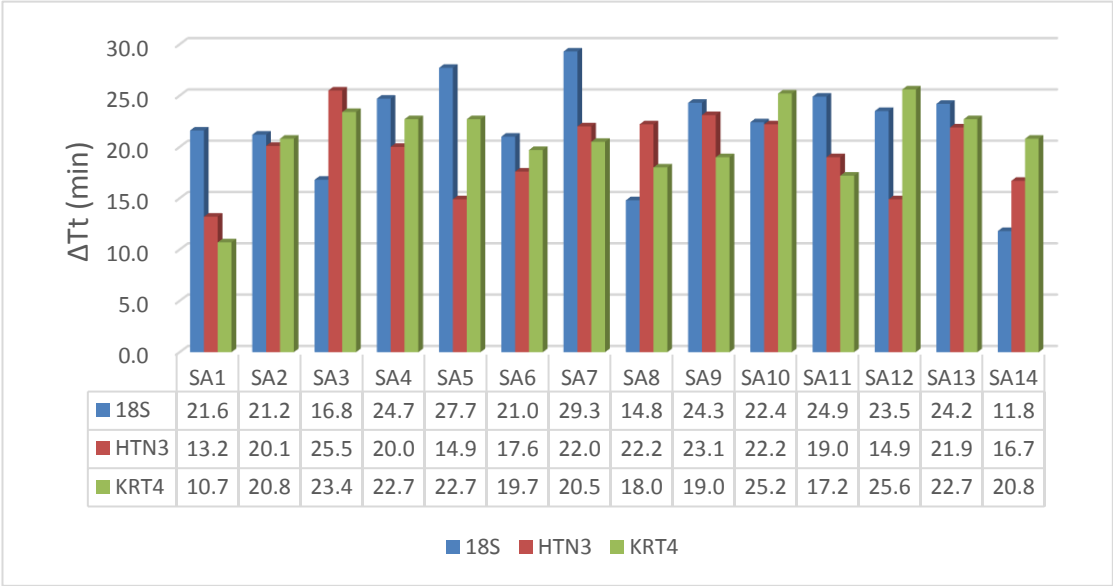
Figure 4-5 The result of the threshold time of RT-LAMP reaction for different body fluids in *KRT4* marker

Different symbols represented the threshold time for each sample. A symbol in the 0 line represented that no LAMP product was detected by the real-time turbidimeter within 60 minutes.

Furthermore, the specificity of markers for each body fluid was also estimated. To clarify the results for the substantial data analysis, ΔTt was calculated as the time between the threshold time and the cut-off time (60 minutes). In other words, ΔTt equals 60 minus threshold time. A greater ΔTt value indicates that the LAMP product reached the threshold earlier and represents more target RNA. The results of real-time RT-LAMP reaction using different primer sets (*18S rRNA*, *HTN3*, and *KRT4*) to different body fluids are shown in from Figure 4-6 to Figure 4-12.

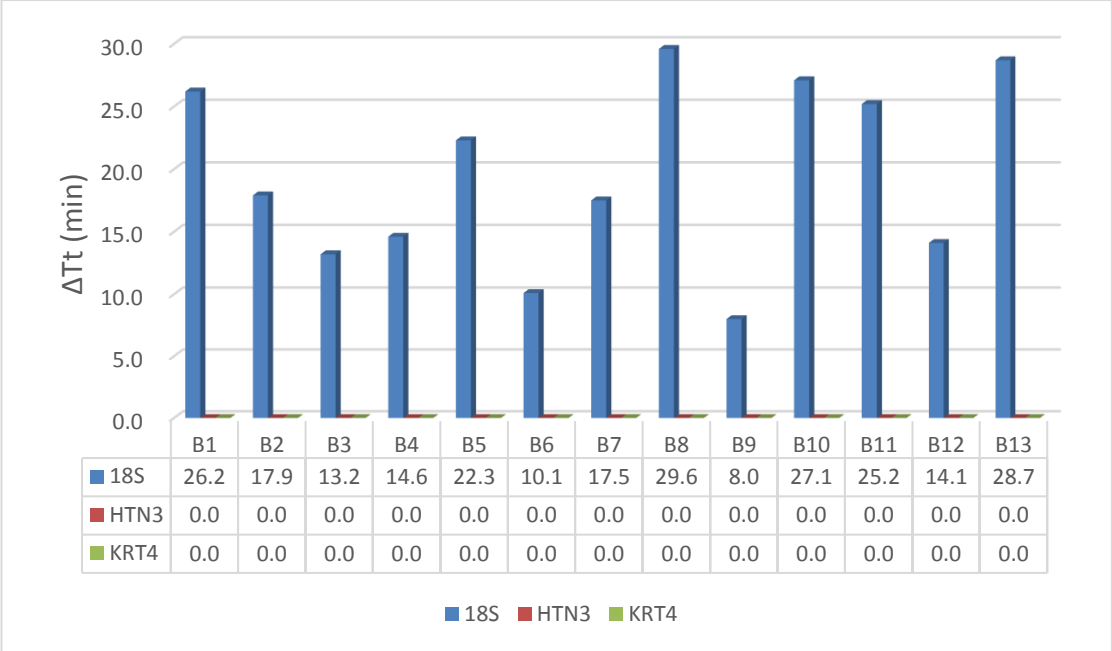
All saliva samples could be successfully identified as positive with both *HTN3* and *KRT4* markers as expected (Figure 4-6). In blood samples (Figure 4-7) and sweat samples (Figure 4-8), no LAMP product was detected with both *HTN3* and *KRT4*

markers within the cut-off threshold time (60 minutes). It indicated that blood and sweat can be successfully identified as not saliva with the two markers. However, in semen samples (Figure 4-9), one sample (sample 7) was found positive with *KRT4* marker. The result was repeated with the same semen sample for three times and was confirmed subsequently as positive.



	SA1	SA2	SA3	SA4	SA5	SA6	SA7	SA8	SA9	SA10	SA11	SA12	SA13	SA14
18S	21.6	21.2	16.8	24.7	27.7	21.0	29.3	14.8	24.3	22.4	24.9	23.5	24.2	11.8
HTN3	13.2	20.1	25.5	20.0	14.9	17.6	22.0	22.2	23.1	22.2	19.0	14.9	21.9	16.7
KRT4	10.7	20.8	23.4	22.7	22.7	19.7	20.5	18.0	19.0	25.2	17.2	25.6	22.7	20.8

Figure 4-6 The result of RT-LAMP for saliva samples using different markers (18S, HTN3, and KRT4)



	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13
18S	26.2	17.9	13.2	14.6	22.3	10.1	17.5	29.6	8.0	27.1	25.2	14.1	28.7
HTN3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
KRT4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Figure 4-7 The result of RT-LAMP for blood samples using different markers (18S, HTN3, and KRT4)

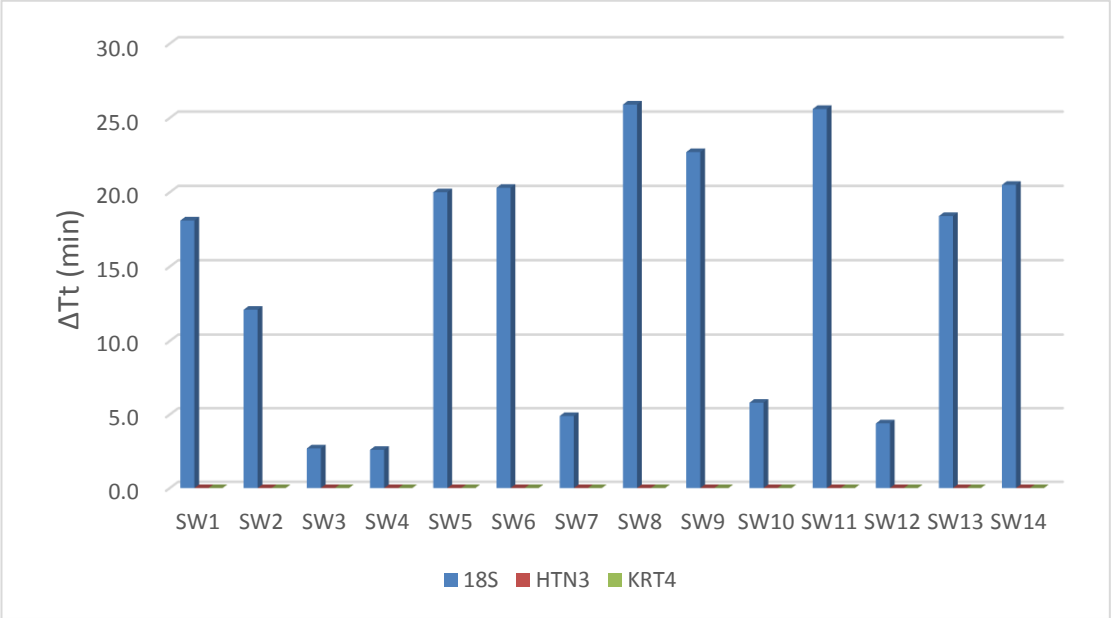


Figure 4-8 The result of RT-LAMP for sweat samples using different markers (18S, HTN3, and KRT4)

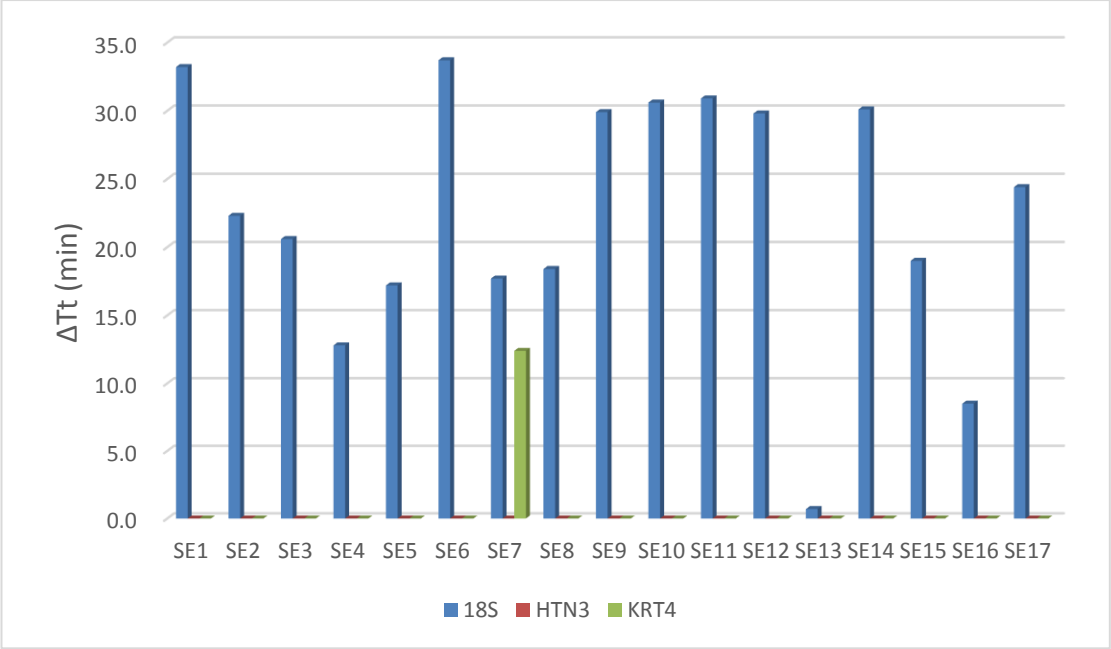


Figure 4-9 The result of RT-LAMP for semen samples using different markers (18S, HTN3, and KRT4)

Besides, in menstrual blood (Figure 4-10), urine (Figure 4-11), and vaginal secretion (Figure 4-12) samples, more than half (7 out of 12 menstrual blood samples, 8 out of 12 urine samples, and 7 out of 15 vaginal secretion samples) were found as positive with the *KRT4* marker, which was not as predicted. This could be explained with the study of Zubakov *et al*., which showed that *KRT4* expression was much less in semen than in saliva [262] and the study of Lindenbergh *et al*., which found the expression of *KRT4* in the vaginal secretion [148]. *KRT4* was also found in urine samples because *KRT4* might be found in the epithelial cells of the esophagus, nasal cavity or even genital. Both semen and urine may contain the epithelial cells of the urethra, but a large portion of the composition of urine is mainly water, so the RNA extraction from urine was mostly acquired from the supernatant after centrifugation. The amount of epithelial cells can be concentrated from this and cause a positive result in LAMP reaction. Besides, the difference of expression between individuals could also be a factor of the variance in the results.

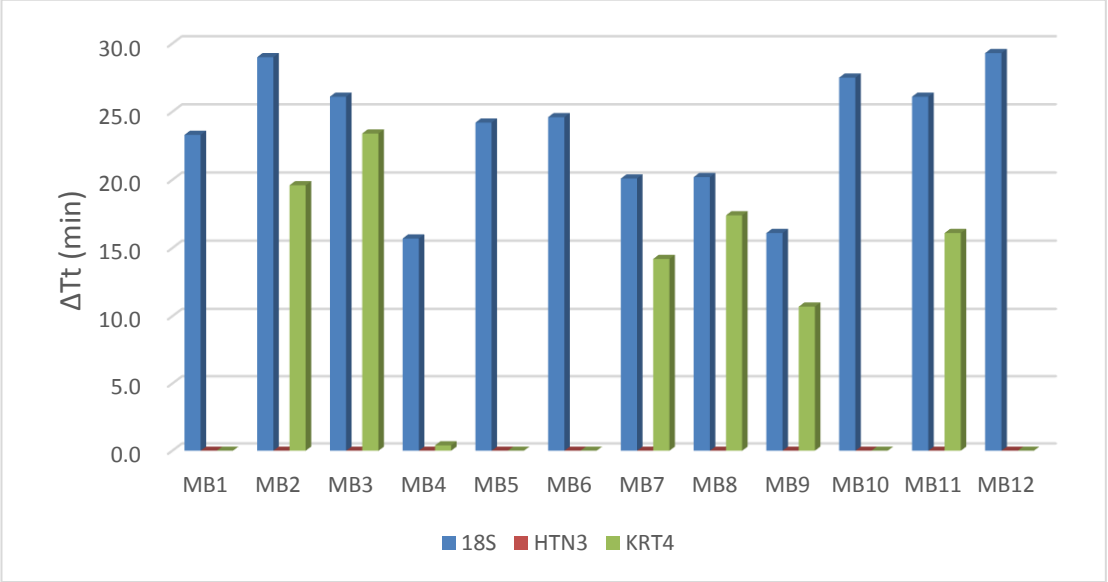


Figure 4-10 The result of RT-LAMP for menstrual blood samples using different markers (18S, HTN3, and KRT4)

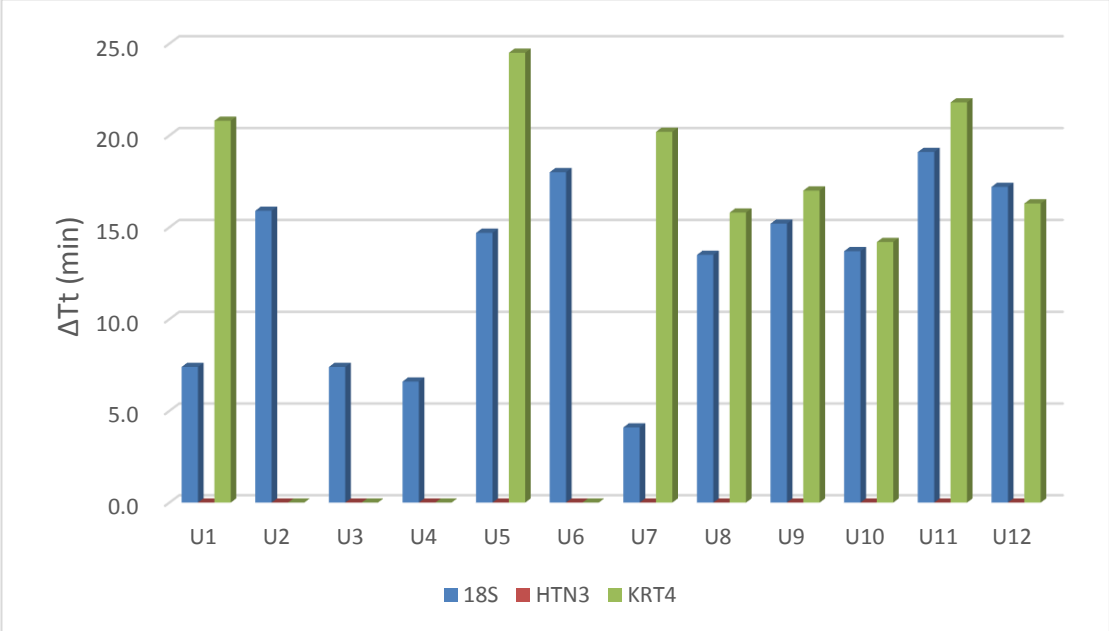


Figure 4-11 The result of RT-LAMP for urine samples using different markers (18S, HTN3, and KRT4)

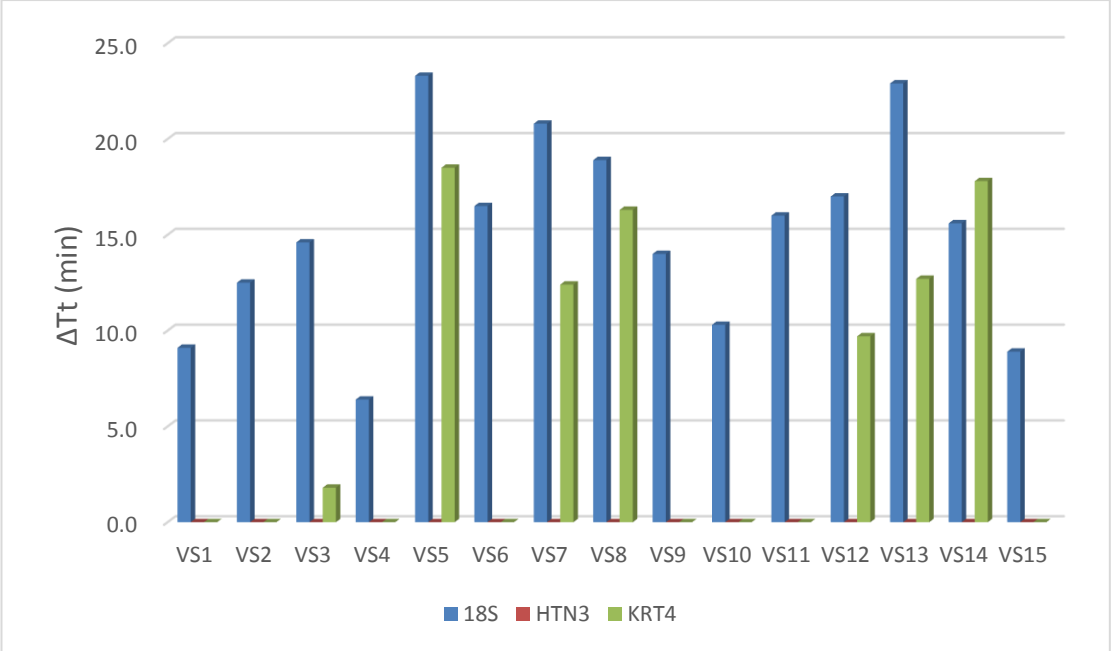


Figure 4-12 The result of RT-LAMP for vaginal secretion samples using different markers (18S, HTN3, and KRT4)

4.3.3 Statistical analysis

Statistical analysis with binary classification of sensitivity and specificity was performed for further evaluation of both markers. The result of saliva samples was compared with the result of the other body fluids by binary classification.

4.3.3.1 Sensitivity and specificity of *HTN3* marker

First, the test outcomes of 14 saliva samples and 83 non-saliva samples were listed in Table 4-9. Based on this table, the statistical measures of the performance (see Section 4.2.6) of the *HTN3* marker (Table 4-10) could be calculated. Since all saliva samples were identified as positive and all the non-saliva samples were correctly identified as negative, all the statistical measures of the performance of the *HTN3* marker were perfect as expected. Nevertheless, the sample size might be too small and more samples are required for further confirmation of its specificity.

Table 4-9 The binary classification for saliva and non-saliva samples in RT-LAMP result based on *HTN3* marker

		Condition (body fluid)							
		Saliva (14 samples)	Non-saliva (83 samples)						Total
			Blood	Semen	Menstrual blood	Sweat	Urine	Vaginal secretion	
Test outcome	*HTN3* positive	14	0	0	0	0	0	0	14
	HTN3 negative	0	13	17	12	14	12	15	83
	total	14	13	17	12	14	12	15	97

Table 4-10 Comparison of the RT-LAMP result between saliva and the other body fluids with *HTN3* marker

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	LR+*	LR-#
Saliva v blood	100	100	100	100	100	NA	0
Saliva v semen	100	100	100	100	100	NA	0
Saliva v menstrual blood	100	100	100	100	100	NA	0
Saliva v sweat	100	100	100	100	100	NA	0
Saliva v urine	100	100	100	100	100	NA	0
Saliva v vaginal secretion	100	100	100	100	100	NA	0
Saliva v all the other body fluids	100	100	100	100	100	NA	0

* LR+ is calculated as Sensitivity/(1-Specificity). However, since the specificity=1, the denominator of the formula equals 0. This makes a condition of division by 0, which has no meaning in mathematics.

LR- is calculated as (1-Sensitivity) / Specificity. Because sensitivity=1, the value of LR- equals 0. This means that the chance to get a negative result from a saliva sample is zero.

4.3.3.2 Statistical sensitivity and specificity of *KRT4* marker

The *KRT4* results of 14 saliva samples were compared with the other non-saliva body fluids (83 samples). The binary classification of the result of saliva and non-saliva samples was shown in Table 4-11 and the sensitivity, specificity, PPV, NPV, accuracy, and likelihood ratios were calculated as shown in Table 4-12.

Table 4-11 The binary classification for saliva and non-saliva samples in RT-LAMP result based on *KRT4* marker

		Condition (body fluid)							
		Saliva (14 samples)	Non-saliva (83 samples)						Total
			Blood	Semen	Menstrual blood	Sweat	Urine	Vaginal secretion	
Test outcome	*KRT4* positive	14	0	1	7	0	8	7	37
	KRT4 negative	0	13	16	5	14	4	8	60
	total	14	13	17	12	14	12	15	97

Table 4-12 Comparison of the RT-LAMP between saliva and the other body fluids with *KRT4* marker

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	LR+*	LR-#
Saliva v blood	100	100	100	100	100	NA	0
Saliva v semen	100	94.12	93.33	100	96.77	17	0
Saliva v menstrual blood	100	41.67	66.67	100	73.08	1.71	0
Saliva v sweat	100	100	100	100	100	NA	0
Saliva v urine	100	33.33	63.64	100	69.23	1.5	0
Saliva v vaginal secretion	100	53.33	66.67	100	75.86	2.14	0
Saliva v all the other body fluids	100	72.29	37.84	100	76.29	3.61	0

* LR+ is calculated as Sensitivity/(1-Specificity). However, since the specificity=1 (for saliva v blood and saliva v sweat), the denominator of the formula equals 0. This makes a condition of division by 0, which has no meaning in mathematics.

\# LR- is calculated as (1-Sensitivity) / Specificity. Because sensitivity=1, the value of LR- equals 0. This means that the chance to get a negative result from a saliva sample is zero.

Compared saliva with blood and sweat samples, all the measures showed perfect results just like the *HTN3* marker. In the comparison between saliva and semen samples, although the sensitivity was 100%, the specificity decreased slightly to 94.12%. The same tendency was observed in PPV and NPV. To evaluate the performance of a test by the likelihood ratio, Sackett *et al.* represented a table (Table 4-13) which can be used for the interpretation of the likelihood ratio [326]. The LR+ for saliva versus semen was 17, and based on Table 4-13, it was strong evidence to say that a given sample is saliva if we got a positive result from the given sample which was alleged to be saliva or semen. However, compared saliva with menstrual blood, urine, and vaginal secretion samples, the results were worse. The values of specificity were 41.67%, 33.33%, and 53.33% respectively. This meant the false positive rates (1-specificity) for menstrual blood, urine, and vaginal secretion samples were 58.33%, 66.67%, and 46.67% in sequence. The LR+ for saliva versus the three body fluids were only 1.71, 1.5, and 2.14 respectively, which provided weak evidence to confirm the sample as saliva or even no significant evidence for the identification.

Table 4-13 The interpretation of the value of a likelihood ratio

Likelihood ratio	Interpretation
>10	Strong evidence to rule in disease
5-10	Moderate evidence to rule in disease
2-5	Weak evidence to rule in disease
0.5-2	No significant change in the likelihood of disease
0.2-0.5	Weak evidence to rule out disease
0.1-0.2	Moderate evidence to rule out disease
<0.1	Strong evidence to rule out disease

Finally, the results for all samples were put together to evaluate the *KRT4* marker for the identification of saliva. Since all saliva samples were correctly identified as positive (no saliva sample was identified as negative), the sensitivity still maintained at 100%. However, the specificity was only 72.29% because 23 samples were wrongly identified as positive among 83 non-saliva samples. This meant that the false positive rate reached 27.71%, which made it risky to apply the *KRT4* marker to saliva identification. Concerning PPV, PPV for all samples (37.84%) was much lower than PPV for saliva versus any single body fluid (more than 63.64%). As mentioned in Section 4.2.6, PPV and NPV relate to the prevalence. The proportion of saliva and non-saliva samples related to the prevalence. If more non-saliva samples are collected for the measurement, PPV decreases dramatically with the change of prevalence, indicating that *KRT4* is not a good marker according to this evaluation. The LR+ measure (3.61) provided only weak evidence to support a real saliva positive result based on Table 4-13.

To summarise, the *KRT4* marker only provided a weak statistical power for the identification of saliva. However, if it is a scenario that an alleged fluid is considered as either a saliva, blood, or sweat sample, then the *KRT4* marker still can be useful for the identification of saliva. For example, a red stain, which is thought to be a bloodstain or a betel nut juice stain which contains saliva, was found at the crime scene. The *KRT4* marker may help the forensic analyst to confirm the type of the evidence for such a scenario. Although the species of body fluids found at the crime scenes are usually unknown, sometimes the assumption can be limited to several types of body fluids due to the other circumstantial evidence or the presumptive tests.

4.3.4 Limit of detection of saliva identification by RT-LAMP reaction

To determine the limit of detection of the assay, 4-fold serial dilutions of total RNA (from 20ng to 0.0078ng) extracted from 3 saliva samples were used and each dilution was tested three times with *HTN3* and *KRT4* markers respectively. For the *HTN3* marker (Table 4-14), both original concentration (20ng) and $1/4^1$ X dilution (5ng) of total RNA extracted from all the three saliva samples tested in triplicate could be detected by RT-LAMP within 60 minutes, except one test of the third sample. In the $1/4^2$ X dilution (1.25ng), all the three tests were positive for the first sample but only one test was positive in the second and third sample respectively. Accordingly, it was suggested that the limit of detection of RT-LAMP is between 1.25 to 5ng total RNA with *HTN3* marker.

Nevertheless, a confusing phenomenon was noticed. The threshold time should increase gradually along with the dilution since the amplification should reflect the amount of the starting materials. However, the values of the threshold time of three positive results for *HTN3* (including test no. 1 of the first sample in $1/4^2$X dilution, test no. 1 of the second sample in $1/4^1$X dilution and test no. 1 of the third sample in $1/4^1$X dilution) did not support this. Besides, although most samples tested negative with *HTN3* marker after $1/4^3$X dilution (0.31ng), it was still found two samples with positive result, one in the third sample with $1/4^3$X dilution and the other in the first sample with $1/4^4$X dilution.

There were several factors that might cause this phenomenon. Firstly, the amount of total RNA, instead of *HTN3* or *KRT4* mRNA, was utilised as the reference amount of the template. Although the selected tissue-specific marker (*HTN3* mRNA in this test) is thought to be expressed in relatively higher level in saliva than in the other

body fluids, it does not mean that it is expressed in an absolutely high level compared with the other RNAs. As mRNA comprised only 1% to 5% of total RNA [131], the proportion of the target mRNA was even less. Although a high amount of total RNA was added in the reagent, it only contained a low proportion of target mRNA as amplification template. As a consequence, a lower amount of template might cause stochastic effects. Stochastic variation is a fundamental physical law of the PCR amplification process. For high copy number DNA, the stochastic effects can be ignored, but the influence increases significantly when low amounts of templates are applied in the PCR reaction [327]. Stochastic effects can play a major role in false positive results especially when the random sampling effects occur in the early cycles of PCR amplification. It may result in artefacts and allele imbalance in STR analysis. The same effects impact LAMP reaction as well since the LAMP is also a reaction of DNA amplification involving primer annealing. Several studies have reported the inconsistency in results obtained when testing samples at the limit of detection using the same molecular protocol [328, 329]. Stochastic effects may also occur with the random sampling from pipetting during the serial dilution and adding of template. Besides, mis-annealing might occur when primers with a lower specificity to the target were used for an isothermal amplification and might also exhibit template free amplification which could lead to false positive results [330].

For *KRT4* marker, the results were similar with *HTN3* marker. Most of the tests before the $1/4^2$ dilution of total RNA (1.25ng) from saliva samples could be detected by RT-LAMP within 60 minutes except that only 2 tests of the second sample in $1/4^2$X dilution were tested as negative (Table 4-15).

Based on the results, it is suggested that the limit of detection of RT-LAMP is around 1.25ng total RNA with *KRT4* marker. Slightly inconsistent threshold time was

found in only one test (the test no. 2 of the second sample in 1/4[1]X dilution), which could be explained by the previous reasons. The limit of detection for the *KRT4* marker (1.25-5ng) was roughly equal to that of the *HTN3* marker (around 1.25ng) based on the tests.

In the preliminary test in Section 3.3.1.1, the average concentration of total RNA extracted from 50µl saliva samples was around 5.3ng/µl and the final elution was 30µl, so the yield of total RNA extracted from the saliva in this study was around 3.2ng per microlitre of saliva. The result showed that less than 1µl of saliva could still be detected using RT-LAMP with the *HTN3* or *KRT4* primer sets and conditions used in this study. Since RNA was extracted from 50µl of starting material, the limit of detection is around 1/50X dilution.

Comparing with other studies involving the identification of saliva using mRNA, the limit of detection of this assay to identify saliva shown in this study (less than 1µl of saliva) is slightly better than that in the study of Roeder *et al*. which aimed to identify body fluids by mRNA profiling (1µl of saliva) [146]. Moreover, RT-LAMP reaction used in this study is faster and more convenient than PCR and capillary electrophoresis used for mRNA profiling. Thus, saliva identification using RT-LAMP is considered better than mRNA profiling based on this study.

As to other presumptive methods used for saliva identification, several techniques have been used in a forensic application (see Section 1.1.3). Among these techniques, Phadebas® Amylase test is widely used. Although the activity of salivary α-amylase may vary due to several factors, such as momentary stress [331], it is still popular because of its sensitivity, convenience and simplicity. According to the study made by Myers *et al.*, the limit of detection of Phadebas® is equivalent to 1:75 dilution

of neat saliva [332], while it is 1:50 diluation in our assay. The limit of detection of our assay is roughly at the same level with Phadebas® Amylase test. However, other body fluids than saliva also can react using the Phadebas® Amylase test and cause false positives [333], which is not found in our assay.

Rapid stain identification of human saliva (RSID™-Saliva), a lateral flow immunochromatographic strip test, is also popular in forensic practice [334] and therefore is also compared here. According to its technical information and protocol sheet, the detection limit for RSID™-Saliva, used as suggested is less than 1µl of human saliva [335], which is similar to our assay. However, Casey *et al.* represented that in cases of several body fluids, such as sweat, urine, and semen, it might result in false positive using the RSID™-Saliva test [336]. Furthermore, low level detect of breast milk and human faecal samples were also observed using RSID™-Saliva [335]. Hence, compared with the RSID™-Saliva test, the specificity of our assay with *HTN3* marker is better.

To sum up, for identification of saliva, RT-LAMP assay with *HTN3* marker is considered at roughly the same level with the current methods routinely used or under development, including the Phadebas® Amylase test, RSID™-Saliva test, and mRNA profiling, in terms of sensitivity. But in further consideration of specificity and convenience, we believe that our assay is a better method than these methods for identification of saliva.

Table 4-14 Limit of detection of the HTN3 marker with saliva

Sample	Test no.	Threshold time (min)				
		Dilution (concentration)				
		1X (20ng)	$1/4^1$ X (5ng)	$1/4^2$ X (1.25ng)	$1/4^3$ X (0.31ng)	$1/4^4$ X (0.0078ng)
1	1	39.4	49.8	44.5	-	53.3
	2	44.7	46.8	48.7	-	-
	3	41.6	49	51.1	-	-
	Avg±S.D.	41.9±2.7	48.5±1.6	48.1±3.3	-	-
2	1	43.5	57.9	53.6	-	-
	2	41.1	59.1	-	-	-
	3	50.6	51.6	-	-	-
	Avg±S.D.	45.1±4.9	56.2±4.0	-	-	-
3	1	40.2	57.0	56.8	43.5	-
	2	40.5	43.1	-	-	-
	3	42.8	-	-	-	-
	Avg±S.D.	41.2±1.4	-	-	-	-

Table 4-15 Limit of detection of the *KRT4* marker in saliva

sample	Test no.	Threshold time (min)				
		Dilution (concentration)				
		1X (20ng)	$1/4^1$ X (5ng)	$1/4^2$ X (1.25ng)	$1/4^3$ X (0.31ng)	$1/4^4$ X (0.0078ng)
1	1	40.0	43.9	52.6	-	-
	2	39.5	44.4	55.4	-	-
	3	40.0	44.0	54.0	-	-
	Avg±S.D.	39.8±0.3	44.1±0.3	54.0±1.4	-	-
2	1	42.0	48.0	-	-	-
	2	43.3	41.4	-	-	-
	3	44.1	46.4	47.2	52.8	-
	Avg±S.D.	43.1±1.1	45.3±3.4	-	-	-
3	1	37.4	40.4	46.6	-	-
	2	36.5	40.0	48.0	59.5	-
	3	37.6	38.8	43.3	-	-
	Avg±S.D.	37.2±0.6	39.7±0.8	46.0±2.4	-	-

4.3.5 Observation of LAMP products

Beside real-time LAMP detection by the real-time turbidimeter, the LAMP products were also detected by two fluorescent dyes, SYBR® Green I and calcein, and electrophoresis. Since the mechanisms of SYBR® Green I and calcein were different, therefore, these two methods were performed separately in different tubes but with the same total RNA. Three light sources, including normal lighting, 450-510nm aquamarine blue light with an orange filter, and UV light with a yellow filter, were applied to observe the results with fluorescent dyes. The results are shown as following (Figure 4-13) and a comparison is made as shown in Table 4-16.

4.3.5.1 Observation of LAMP products with SYBR® Green I

SYBR® Green I was added to the reagents after LAMP reaction and then the LAMP products were observed under three different light sources. The results of *HTN3* and *KRT4* markers were shown in Figure 4-13 (A) and (D), respectively. The first row showed the outcomes of LAMP products of different body fluids with SYBR® Green I added and observed by the naked eye under normal lighting. Concerning the *HTN3* marker, only saliva emitted clear yellow-green fluorescence and was distinguishable from the products of the other body fluids. For the *KRT4* marker, not only the LAMP product of saliva but also that of menstrual blood emitted yellow-green fluorescence. The result of menstrual blood was consistent with the result mentioned in the specificity test and was likely a false positive reaction.

LAMP products with SYBR® Green I were then placed under 450-510nm aquamarine blue light and observed with an orange filter. For the *HTN3* marker, strong yellow-green fluorescence could be observed in the saliva sample and was easily to be distinguished from the samples of the other body fluids. For the *KRT4* marker, both LAMP products of saliva and menstrual blood emitted yellow-green fluorescence. Though the intensity of fluorescence emitted in the saliva and menstrual blood was not very strong, it was still distinguishable from that of the other samples.

Finally, LAMP products with SYBR® Green I were placed under UV light with a yellow filter. Concerning the *HTN3* marker, fluorescence was observed in saliva. The intensity was comparatively weak although it was still distinguishable from that of the other samples. Besides, there was also slight fluorescence observed in the urine sample under the UV light. The intensity of fluorescence in the urine sample is between that in the saliva sample and the negative control, which might cause problem in determining the result of the urine as positive or negative without a positive control reference. For the *KRT4* marker, distinguishable yellow-green fluorescence was observed in saliva and menstrual blood samples, the result of which was consistent with that observed under normal lighting and under 450-510nm aquamarine blue light.

4.3.5.2 Observation of LAMP product with calcein

In contrast to SYBR® Green I, calcein was added to the reagents before performing LAMP reaction. The products with calcein were also observed under three different lighting sources. The results of *HTN3* and *KRT4* markers were shown in Figure 4-13 (B) and (E), respectively.

As for the *HTN3* marker, only saliva emitted yellow-green fluorescence under normal lighting and, though the intensity is rather weak, it was still distinguishable from the samples of the other body fluids. For the *KRT4* marker, similar to the result of SYBR® Green I, both LAMP products of saliva and menstrual blood showed slightly yellow-green fluorescence under normal lighting.

For products of the *HTN3* marker observed under 450-510nm aquamarine blue light with an orange filter, clear yellow-green fluorescence was emitted in the saliva sample, which could distinguish the saliva sample from the others. For the *KRT4* marker, products of saliva and menstrual blood showed yellow-green fluorescence.

For products of the *HTN3* marker observed under UV light with a yellow filter, although samples of all body fluids except saliva did not exhibit fluorescence, the fluorescence emitted from the saliva sample was so weak that it seemed transparent and could not be identified easily. For products of the *KRT4* marker, products of saliva and menstrual blood emitted distinguishable fluorescence though the intensity was weak.

The results showed that clear distinction between positive and negative samples were observed in the LAMP products using SYBR® Green I or calcein as an indicative dye. Comparing the results of 450-510nm aquamarine blue light and UV light in terms of intensity of fluorescence, the former was a better light source as under which the fluorescence of the positive samples observed was slightly stronger and therefore easier to be identified than the latter.

The results also showed that detection of fluorescence using either fluorescent dye could be carried out under normal lighting with naked eye if there were sufficient products for testing, which implied a practical value to apply this method to on-site

criminal investigation. Comparing SYBR® Green I and calcein, the result of the former was better in terms of intensity of fluorescence emitting from positive samples. However, since it is required to open the lid of the reagent tube to add SYBR® Green I after the reaction, there is a possibility of contamination. Nevertheless, SYBR® Green I is still a good fluorescent indicator for LAMP product detection based on this study.

Although the contrast between positive and negative products is not as clear as SYBR® Green I, calcein can still be used as a fluorescent indicator for LAMP product detection. Moreover, because calcein can be added to reagents before the reaction, no more steps are required further for entire detection. As long as the reagent is prepared properly, the result can be obtained at once after the reaction. Analysts can even check the result by observing the changes of fluorescence while the reaction is still in process. Finally, since there is no need to open the lid of the reagent tube, the possibility of contamination greatly reduces.

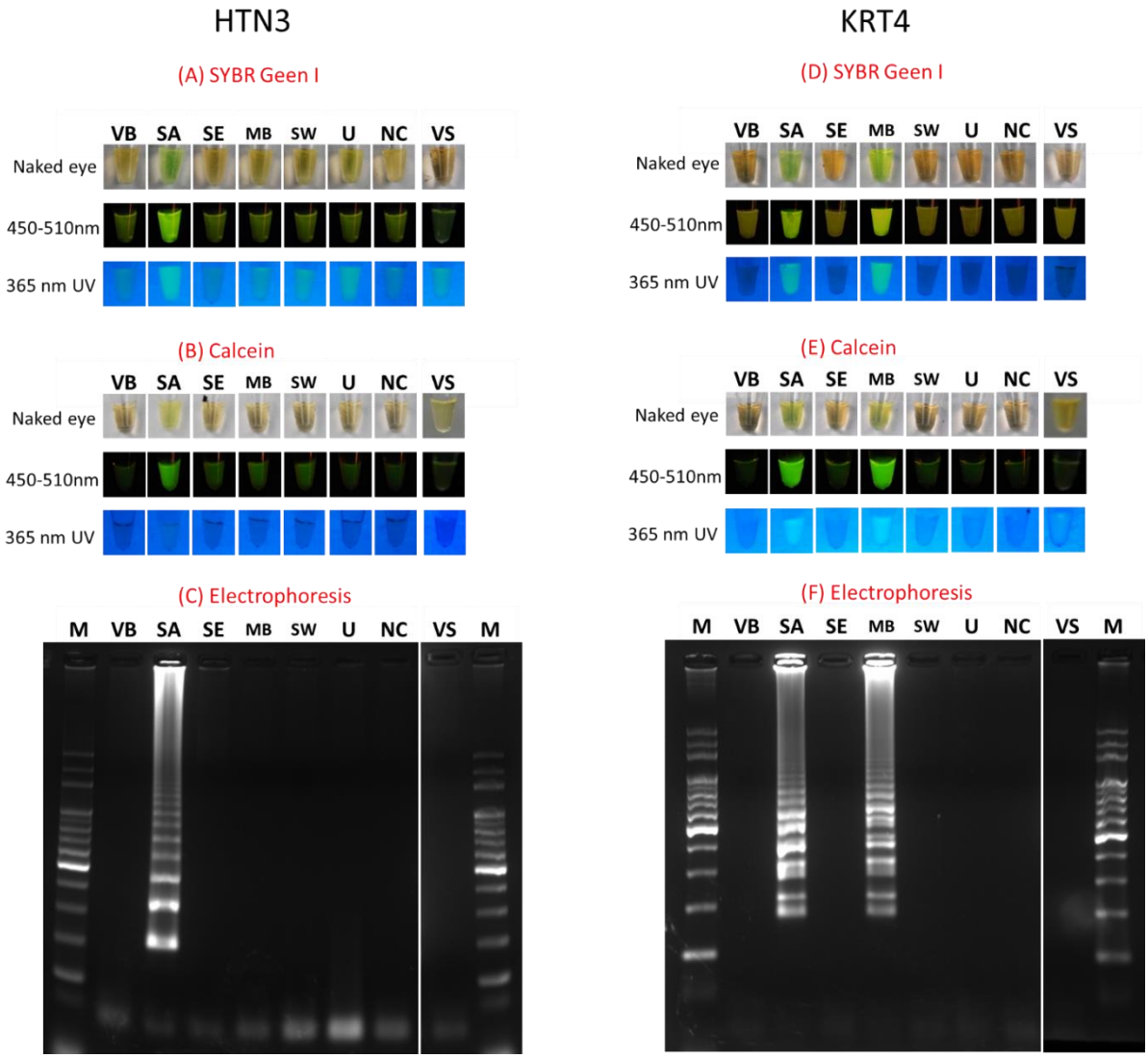


Figure 4-13 Observation of LAMP products for HTN3 and KRT4 markers by SYBR® Green I, calcein fluorescence, and agarose gel electrophoresis

Both LAMP products from HTN3 and KRT4 markers were detected using SYBR® Green I (A and D), calcein (B and E) fluorescence, and agarose gel electrophoresis (C and F). For the observation with SYBR® Green I and calcein fluorescence, 450-510nm aquamarine blue light source (with an orange filter) and 365nm UV (with a yellow filtered filter) were used except observing by the naked eye.
M: 100bp marker; VB: Venous Blood; SE: Semen, SA: Saliva; MB; Menstrual Blood; SW: Sweat; U: Urine; VS: Vaginal Secretions; NC: Negative Control.

Table 4-16 A comparison between different methods of detecting LAMP products from saliva and non-saliva samples.

Method of detecting LAMP products	Positive samples	Non-positive samples
Real-time turbidimeter	+	-
electrophoresis	+	-
SYBR® Green I / the naked eye	+	-*
SYBR® Green I / 450-510nm and filter	+	-
SYBR® Green I / 365nm UV and filter	+	-*
Calcein / the naked eye	+#	-
Calcein / 450-510nm and filter	+	-
Calcein / 365nm UV and filter	+#	-

Positive conclusion could be made by the method but it is sometimes confusing.
* A faint light might be observed when observing.
Positive samples include saliva samples with *HTN3* marker, and saliva and menstrual blood samples with *KRT4* marker.
The comparison is made from the result of *HTN3* and *KRT4* markers. The result of detection using real-time turbidimeter is determined based on the threshold time (see Section 4.3.2). The results of the other methods is determined by the naked eye.

4.3.5.3 The influence of calcein on RT-LAMP

Unlike SYBR® Green I which was added after the reaction and therefore would not affect the LAMP reaction, calcein was added in the reagent mixture before the LAMP reaction and might impact the reaction. Thus, the effect of calcein on the LAMP reaction was also tested in this study. Real-time RT-LAMP was carried out with 6 saliva samples using *18S rRNA* marker. For each saliva sample, RT-LAMP reaction was performed with/without calcein respectively. The threshold time was 39.5±1.6 minutes for the reaction without calcein and 45.3±2.4 minutes for the reaction with calcein. The difference of threshold time between the reactions with/without calcein was 5.8±1.5 minutes. Figure 4-14 is the box plot of the threshold time for RT-LAMP

reaction with/without calcein, which shows obvious difference ($t=4.93 > t_{12,0.025}=0.18$). Besides, real-time RT-LAMP reaction with or without calcein added in advance was also tested using different body fluids. The difference of threshold time among different body fluids was between 5.6-13.3 minutes (Table 4-17). The results showed that the threshold time for real-time RT-LAMP reaction increased when calcein was added to the reagent mix in advance, so the use of calcein is not recommended when the sample is tested by real-time RT-LAMP. Nevertheless, calcein could still be useful when an end-point RT-LAMP is performed since the result can be obtained at once after the reaction by fluorescent observation without further operation. Besides, it also prevents contamination because there is no need to open the lid of the reaction tube.

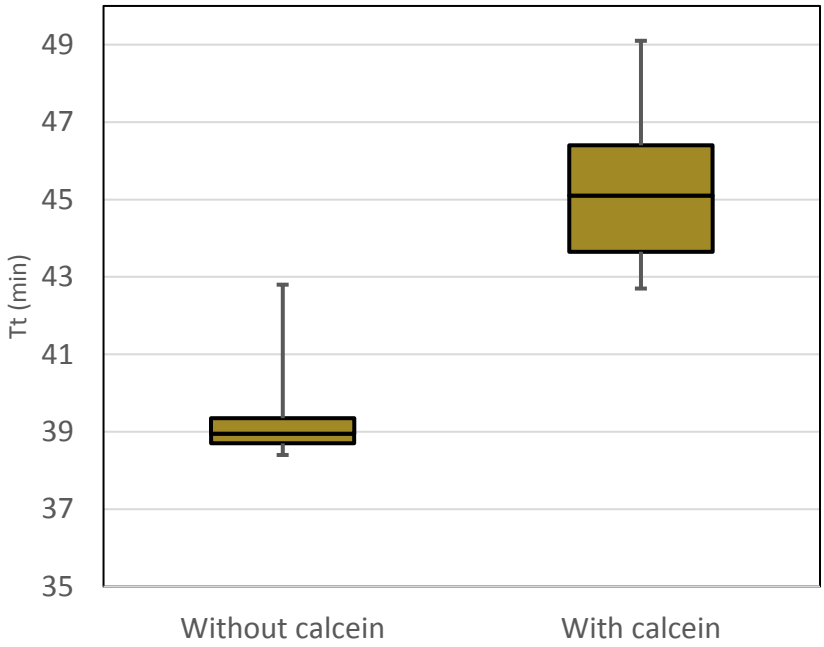


Figure 4-14 Box plot showing the difference of threshold time between RT-LAMP with/without calcein

Six saliva samples were tested using 18S rRNA marker to examine the influence of calcein for RT-LAMP. For each saliva sample, RT-LAMP reaction was performed with/without calcein respectively. The top of the rectangle indicates the third quartile, a horizontal line near the middle of the rectangle indicates the median, and the bottom of the rectangle indicates the first quartile. A vertical line extends from the top of the rectangle to indicate the maximum value, and the other vertical line extends from the bottom of the rectangle to indicate the minimum value.

Table 4-17 Comparison of the threshold time with/without calcein using different body fluids

	Blood	Saliva	Semen	Menstrual blood	Sweat	Urine	Vaginal secretion
Threshold time of RT-LAMP without calcein (min)	+ (30.4)	+ (45.2)	+ (41.6)	+ (39.8)	+ (34.1)	+ (46.5)	+ (38.8)
Threshold time of RT-LAMP with calcein (min)	+ (43.7)	+ (51.0)	+ (47.2)	+ (46.9)	+ (39.7)	+ (58.0)	+ (44.6)
Difference (min)	13.3	5.8	5.6	7.1	5.6	11.5	5.8

4.3.6 Detection and confirmation of RT-LAMP products by electrophoresis

The presence of LAMP products was confirmed by electrophoresis in this study. Clear bands were observed in positive samples (Figure 4-13 (C) and (F)). The result was observable with the naked eye initially and confirmed by electrophoresis without additional steps.

For *HTN3* marker, clear ladder-like bands were only found in the LAMP product of saliva sample (Figure 4-13 (C)). Weak fluorescence was observed in urine sample under UV light in the previous fluorescent test but no amplification was detected here. For *KRT4* marker, clear ladder-like bands were observed in the LAMP product of saliva and menstrual blood samples (Figure 4-13 (F)), which was consistent with the previous fluorescence test. Comparing all the fluorescence and electrophoresis results, the fluorescence test could be subjective and might raise the possibility of false result. Besides, it was noticed that the bands of LAMP fragments in the gel from electrophoresis could be easily observed while the fluorescence from calcein or SYBR® Green I was equivocal in a few samples. Thus, electrophoresis was considered a better

method for detecting LAMP products. However, as electrophoresis is an end-point detection for the LAMP product, the reaction cannot be monitored in real-time.

4.4 Discussion

In this study, *18S* rRNA was selected as a standard control of body fluid identification and 2 specific markers, *HTN3* and *KRT4* mRNA, were tested for the identification of saliva by RT-LAMP. The result of specificity and sensitivity tests showed that *HTN3* was only found in saliva and could serve as a good candidate marker for identification of saliva. As to *KRT4*, although it showed perfect specificity for saliva when comparing with blood and sweat samples, it was not reliable when applied to menstrual blood, urine, and vaginal secretion samples. Further estimation of both markers was performed using statistical analysis with a binary classification. The *HTN3* marker provided strong support for the confirmation of saliva, while the *KRT4* marker exhibited only a weak statistical power and a relatively high false positive rate for saliva identification. Similar results were also found in previous studies made by Zubakov and Lindenbergh *et al* [148, 262]. Although the *KRT4* findings were not as expected, the *KRT4* maker might still be useful in some scenarios when an alleged body fluid is saliva or blood. For example, betel nuts are prevalent in the countryside of Taiwan. People who chew a betel nut may spit betel nut juice (mixed with saliva) after chewing. Betel nut juice is often found at crime scenes, especially in burglary cases, and its colour is dark red, which is similar to blood. In this case, it is crucial to identify whether an alleged fluid is blood or betel nut juice with saliva. The *KRT4* marker can be useful in such a scenario. Besides, the *KRT4* marker can also be used together with the *HTN3* marker to improve the reliability of saliva identification.

Variation amongst samples was found not only in the specificity test but also in the evaluation of the limit of detection of both *HTN3* and *KRT4* markers. The variations of Tt values among saliva samples might result from differences in RNA degradation, progress of RNA extraction, and quantification. Besides, as mRNA only comprises a small portion of total RNA, a slight change in the amount of the target mRNA might cause the variation of the result. Moreover, the level of RNA expression can be highly variable in different individuals and tissues. It may change over time in the same body fluid of the same person due to physiological or psychological conditions. As a result, it might be arbitrary to confirm the body fluids with only one specific marker. To overcome this, at least five specific markers are suggested to be used to confirm a body fluid [146].

Comparing *HTN3* and *KRT4* markers with *18S* rRNA in terms of sensitivity, *18S* rRNA could be detected even only with 10^{-5}ng total RNA (reported in Chapter 3) but the limit of detection of RT-LAMP was between 1.25ng to 5ng of total RNA with *HTN3* marker and around 1.25ng with *KRT4* marker. The difference might result from several factors. First of all, as mentioned previously, mRNA comprises only 1% to 5% of total RNA and rRNA comprises about 80% of total RNA in a cell. There are approximately 360,000 mRNA molecules in a single cell, containing approximately 20,000 to 30,000 different mRNA species. A single cell may contain only a small amount of a given specific mRNA. In this study, the amount of total RNA was used as a reference amount for serial dilution in the detection of limitation. A certain amount of total RNA may contain a large number of rRNAs and mRNAs, but only a low amount of target mRNA might be present and could be used as template, despite that it is relatively highly expressed in saliva. Comparatively, rRNAs are expected to be more robust than mRNAs due to their protective protein complex that shield them from degradation

through environmental impacts (see Section 5.1.3). In addition, the source of *HTN3* or *KRT4* mRNA in saliva is considered from the epithelial cells of salivary glands, while 18S rRNA can be found in most cells. As a consequence, *18S* rRNA may still be detectable while *HTN3* and *KRT4* mRNA can no longer be detected.

In this study, four different methods for detection of the RT-LAMP products were used, including real-time turbidimeter, SYBR® Green I, calcein, and electrophoresis. Comparisons of these methods' advantages and shortcomings are listed as Table 4-18.

Firstly, in regards to the two fluorescent dyes, using calcein as the indicator is more convenient than SYBR® Green I since it is added to the reagent mix before the reaction. The shortcoming of using calcein is that it affects the subsequent RT-LAMP reaction. Comparing the RT-LAMP reaction with/without adding calcein, it is noticed that the Tt value for real-time RT-LAMP reaction increased significantly when calcein was added to the reagent mix in advance. This phenomenon was also noticed by Fischbach *et al*. [337]. The Tt value increases due to the manganese contained in the calcein-based dye, which is known to inhibit the LAMP reaction and might reduce the overall sensitivity [338] and thereby produce a false negative when only a small amount of saliva is tested. Using SYBR® Green I as the indicator does not affect the reaction at all since it is added after the reaction. However, amplified LAMP product might be volatile and easy to contaminate each other. It requires the opening of the lid of the tube to add SYBR® Green I after reaction, raising the risk of contamination for the next analyses.

In conclusion, both SYBR® Green I and calcein are capable of detecting the LAMP product. However, the fluorescence from SYBR® Green I or calcein might be

undetectable in some samples as confirmation of the LAMP products with the naked eye may be affected by many factors, such as the subjective judgement of the scientist and the lighting conditions. Thus, it is suggested that positive and negative controls should always be included and used for comparison. The use of SYBR® Green I or calcein may not always accurately distinguish true positives from false positives. Presumptive tests could be performed in advance and then followed up with LAMP tests to prevent false results. Electrophoresis could be used to confirm the result if it is not clear with SYBR® Green I and calcein.

Regarding the required devices for reaction of the four methods, the real-time turbidity requires a real-time turbidimeter, while the other three methods only require a thermal cycler or a simple heater. As to the required devices for detection, the real-time turbidimeter is all set for the detection of the turbidity, and electrophoresis requires the gel and an electrophoresis tank. Detection by SYBR® Green I or calcein can be done by the naked eye with normal light source although specific light sources and filters will strengthen the contrast between the positive and negative samples.

In terms of convenience, the real-time turbidity is the most convenient method since the instrument can complete the whole analysis and provide an objective final result, without the subjectivity added by human observation. Both SYBR® Green I and calcein are also easy to use. Nevertheless, as SYBR® Green I is added after the reaction finishes, it takes more time. As for electrophoresis, it takes more time to load the sample to gel, conduct electrophoresis and photo the result. Thus, compared with the other three methods, it is less convenient for detecting the LAMP product.

Cost and effectiveness are also evaluated in this study. The cost for each reaction of each method is roughly the same because the main cost required is for the RT-LAMP kit. There is no specific instrument required other than a thermal cycler or a simple heater for conducting fluorescence analysis. As for the other methods, a real-time turbidimeter is required to perform real-time RT-LAMP, and an electrophoresis tank is required for electrophoresis. In Table 4-18, only the cost of each reaction was compared, and the cost for required equipment was not taken into account. As to the effectiveness, the time to complete the entire reaction is 60 minutes for all reactions, except electrophoresis, which requires additional 30 minutes.

Table 4-18 Comparisons between 4 methods for confirmation of LAMP product in this study

	Real-time turbidity	SYBR® Green I	Calcein	Electrophoresis
Risk of contamination	Low	High	Low	High
Required device for reaction	Real-time turbidimeter	Thermal cycler or heater	Thermal cycler or heater	Thermal cycler or heater
Required device for detection	Real-time turbidimeter	N/A	N/A	electrophoresis tank
Judgment	Setting on the device	Man-made	Man-made	Man-made
Sensitivity	Normal	Better	Better	Best
Convenience	Best	High	High	Medium
Price per reaction (pound)	3.18	3.23	3.75	3.83
Time for whole reaction (minute)	60	60	60	90

Although our study revealed that the two markers, *HTN3* and *KRT4*, can be used to identify saliva, its results should be treated with reservation, owing to the number of samples, type of samples, and possible false transcription of mRNA. First, the sample size used in the specificity test was 12 to 17 for each body fluid, which could be too small to evaluate the specificity of a marker. More samples for each body fluid would benefit the statistical power as the smaller the sample size, the higher the margin of error. Second, although seven common body fluids were examined in this study, some other unusual body fluids, such as nasal mucus, were not tested. It might be erroneous to say that the *HTN3* marker is saliva-specific based on the results here. Thus, more body fluids should be collected and further evaluated for the test of specificity. Third, as mentioned in Section 1.3.4, erroneous transcription of mRNA may occur and lead to a false conclusion. To avoid this, additional markers should be used and other identification methods should be considered for further confirmation when the results of LAMP reaction from different markers are inconsistent with each other.

In summary, a novel approach for the identification of saliva is represented in this study. This is the first application of RT-LAMP for the identification of saliva. The use of RT-LAMP showed great potential in forensic casework by presenting the possibility to identify body fluids via mRNA. With the adoption of more markers, this approach could provide forensic scientists with novel probative information from a crime scene stain.

Chapter 5 Evaluation of dating forensic hair samples

Hair is primarily composed of the protein keratin, and it is one of the most common types of biological evidence collected at crime scenes owing to the facts that hairs of a human being shed naturally every day and that hairs are easily removed in a struggle or physical contact such as a violent fight. The average number of hairs on the scalp of an adult human is around 150,000, and there are around 75 to 100 telogen hairs lost every day [339]. Besides, hair is generally very persistent on fabrics or beneath fingernails. Therefore, hair is normally found at crime scenes either from natural shedding or from being plucked during physical contact. Hair evidence could associate a suspect with a crime scene or a victim and also could provide temporal information for the timeline construction of a crime event which is vitally important in criminal investigation. However, hairs at crime scenes are usually ignored in criminal investigation because they are too small to be noticed and collected. Additionally, compared to the other types of biological evidence such as blood, hairs, especially shed hairs, provide less genetic material, such as DNA and RNA, for further forensic analysis due to the fact that the nuclei and protein making machinery is broken down during the formation of these keratin shafts.

In the past decades, numerous studies have been conducted on forensic hair identification [340]. Usually, a two-step preliminary confirmation test of hair identification is conducted before the forensic analysis of hair samples. The first step is to confirm whether or not the sample is a hair sample as fibres might also be misidentified as hairs with the naked eye. The next step is to determine if the hair in question originates from a human being or an animal by microscopy because it is also very common to find animal hairs (such as dog or cat hairs) around human habitations. Microscopic features of the sample, including shape, appearance, width, and length,

can distinguish one species from another. After the preliminary procedures of confirmation, DNA analysis can be performed on forensic hairs. Most studies of hair identification focus on DNA from hair roots owing to the fact that only a small quantity of genomic DNA can be obtained from hair shafts and the extracted DNA from hair shafts is usually degraded, causing difficulty on analysis [341]. The methods usually used for DNA analysis of forensic hair samples are STR profiling on hair roots and mitochondrial DNA (mtDNA) sequencing [342, 343]. Species identification of animal hairs is also presented in several studies [344, 345], which is helpful in determining the animal species of the hair samples.

Beside species identification and DNA analysis, scientists may also try to evaluate the time since the hairs were deposited at the crime scene. An accurate estimate of the age of a plucked hair reveals the time since it was plucked at the crime scene and provides a temporal link with the crime event in criminal investigation. Dating a forensic hair sample is important as it not only reveals the time when the crime occurred, but also helps to build the timeline of a crime which may include or exclude potential suspects.

Anderson *et al.* used RNA quantification to date bloodstains [177, 191] and provided supportive results for the hypothesis that there are differences in *ex vivo* RNA decay rates (*ß-actin* mRNA decays more rapidly than *18S* rRNA). Based on this hypothesis, it is explored to estimate the time since the hair was plucked using RNA quantification and compare the relative amount of the two RNA markers in this study.

5.1 Introduction

5.1.1 The growth cycle of hair

The growth of human hair occurs everywhere on human skin except for certain specific areas, such as soles of the feet, palms of the hands and the lips. The hair is produced by the hair follicle which is a mammalian skin organ. The growth cycle of hairs contains three phases in sequence: anagen phase, catagen phase and telogen phase (Figure 5-1). All three phases occur simultaneously with one strand of hair in the anagen phase and another strand in the catagen or telogen phase. Each hair strand is at its own phase of the cycle. The growth cycle is roughly 3 to 6 years depending on where the hair roots reside.

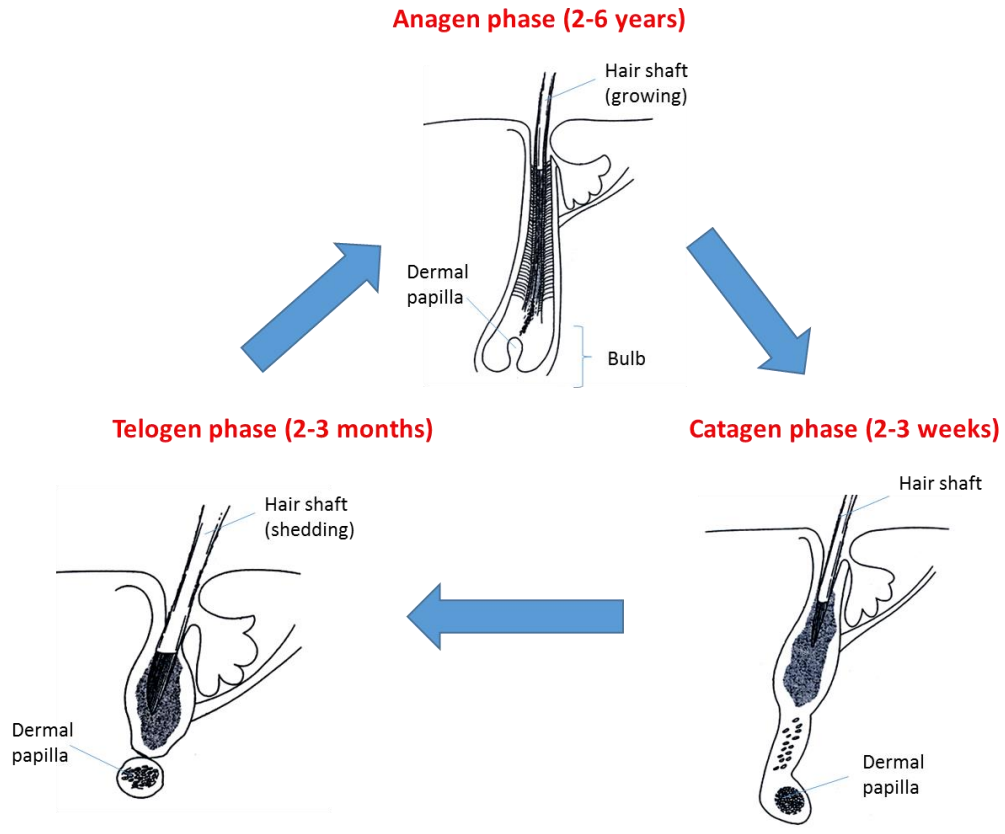


Figure 5-1 Three phases of the hair growth cycle

Each hair phase has its own specific characteristics. The first stage is the anagen phase (also known as growth phase). In this stage, the cells in the papilla (a large structure at the base of the hair follicle) divide to produce new hair fibres. The scalp hair grows about 0.35mm per day and lasts for 2 to 6 years. Approximately 85% to 95% of all hairs are in the growth phase at any given time [346, 347]. The catagen phase (also known as the transitional phase) begins at the end of the anagen phase. It is a short transition stage that lasts for about 2 to 3 weeks. In this stage, the hair follicle stops growing and shrinks. The hair converts to a club hair which has a bulge at the end and the hair shaft is pushed upward by the hair follicle. Only around 1% of scalp hairs are in the catagen phase. The telogen phase begins when the club hair has been formed completely. During the telogen phase, also called the resting phase, the hair follicle is completely at rest. Around 10% to 15% of scalp hairs for an individual are in the telogen phase. The telogen phase lasts about 2 to 3 months, and then the old hair shaft naturally falls off the scalp, resulting in a normal hair loss known as shedding, and a growth cycle of hair completes. The rest hair follicle starts to grow again, and enters the anagen phase. A new cycle starts.

5.1.2 Current methods for identification of hairs

Several methods have been used for hair analysis, including microscopic comparison, analysis of proteins, enzymes, chemicals elements [348, 349], DNA analysis, and so on. Microscopic examination is usually the first step to confirm the source of a hair sample and DNA analysis is then applied for DNA profiling or mitochondrial DNA sequencing. DNA analysis is the most potent tool and has become

the best choice for hair identification. The following is the illustration of these frequently used methods.

5.1.2.1 Microscopic analysis

The examination of human hairs is primarily conducted through microscopic comparison to identify the source of the sample. Hairs can be identified from their physical aspects, such as shape, colour, layers of cuticle as well as distribution of cortex, medulla, melanin and other factors [350, 351]. However, microscopic hair identification is not always reliable as it is subjective and relies heavily on the expertise of the analyst. This professional ability varies from one analyst to another. Hence, microscopic analysis of hair identification might sometimes lead to wrong conclusion. Although some statistical methods, which calculated the statistical likelihood, were applied to evaluate hair comparisons [352-354], no solid answer was provided. To assure the reliability of microscopic analysis, analysts require professional training as well as regular proficiency tests annually thereafter, and on the other hand, standard operating procedures and guidelines must be followed during the entire identification process.

Due to the lack of adequate and objective statistics in most aspects of hair identification (for example: the probability of feature points and the possibility of errors), microscopic results often raise questions. There have been several reports regarding errors caused by microscopic examination [355]. With the improvement of DNA identification technology, microscopic identification for hairs has been substituted by DNA technology and is no longer used in the forensic field [355-357]. Nevertheless, it is still useful for the preliminary examination of hairs.

5.1.2.2 Nuclear DNA identification

DNA typing from single hairs was firstly presented by Higuchi *et al.* in 1988 [358]. Along with the PCR development [206, 359], hair identification by forensic DNA technology is widely used nowadays [360]. During the preliminary microscopic examination of hairs, forensic scientists select hair samples with the hair roots to ensure they have sufficient nuclear DNA for successful DNA profiling [361]. Nowadays the most useful and commonly used method is STR analysis (see Section 2.5.5).

In spite of the advantages of STR technologies, STR technologies still have limitations when applying to identification of human hairs. First, hair fibres are actually the products after cornification by keratinocytes. The process of cornification involves destruction and degradation of the cell nucleus. As a consequence, the hair shaft contains very little nuclear DNA. More than 90% of hairs found at crime scenes are telogenic hairs [362, 363] as hairs in telogen phase shed naturally. DNA in the telogen hairs is usually degraded critically due to dehydration and keratinisation, and the degraded DNA fragments are around 100 bp [364, 365]. Therefore, analysis of nuclear DNA from hairs at crime scenes is challenging for forensic scientists.

To improve the DNA-STR analysis for hairs, Hellmann *et al.* designed a new approach to analyse the DNA extracted from shed telogen hairs [366]. Newly designed primer pairs for STR loci HUMFES, HUMTH01 and HUMTPOX were used for amplification with the aim of reducing the length of the PCR products (less than 106 bp for HUMFES, 86 bp for HUMTH01 and 87 bp for HUMTPOX), thereby increasing the chances of obtaining an STR profile. Therefore, using mini-STR could raise the success rate of identification of hairs [367-371]. Commercial kit is also available for mini-STR

analysis. However, hair samples may not allow the determination of complete DNA profiles even using the mini-STR analysis [372].

5.1.2.3 Mitochondrial DNA identification

Mitochondrial DNA, as the name suggests, is located in the mitochondria. It is organized as a closed-circular double-stranded DNA molecule of 16569 bp and contains 37 genes coding for 2 rRNAs, 22 tRNAs as well as 13 polypeptides [373]. Comparing to the nuclear DNA (only single copy per cell), about 100-10000 copies of mtDNA are usually present in each cell, except egg and sperm cells [374]. Even in highly degraded samples or trace evidence, which may contain only small fragments of degraded genomic DNA, a large number of mtDNA may be still present. Mitochondrial DNA has been proven to be an efficient target in forensic science and also has been widely used in maternity identification owing to its special characteristics such as rapid mutation and maternal inheritance [375]. It can also be used for identification of animal species [344, 345].

Mitochondrial DNA has a high mutation rate (around 10 times higher than nuclear genomic DNA) caused by lack of protective histones, inefficient DNA repair systems, and continuous exposure to mutagenic effects of oxygen radicals [376]. This property makes mtDNA useful in forensic identification. The human mitochondrial hypervariable regions I and II (known as HV I and HVII) of the control region (D-loop region) are often used for mitochondrial DNA sequence comparison [377] (Figure 5-2). Human mitochondrial DNA sequence, also known as “Anderson” or Cambridge reference sequence (CRS), was first identified in 1981, and it has been used as the

standard reference sequence. It was revised in 1999 by Andrews who confirmed that almost all of the originally identified nucleotides are present in CRS [378].

Since it is difficult to identify hairs in the telogen phase with DNA-STR technology owing to the small quantity of good quality DNA, mtDNA sequencing has become the most popular method [379, 380] for this type of hair. Although sequencing provides a higher success rate for hair mtDNA identification, some difficulties still remain. For example, it takes more time and labour for mtDNA sequencing. Moreover, mtDNA is maternally inherited so all female descendants will have the same sequence. In addition, heteroplasmy is sometimes found in the mtDNA [381]. The phenomenon of heteroplasmy may confuse the results. Furthermore, melanin, which is found in hair shaft, binds to thermostable DNA polymerase reversibly, and as a result, it inhibits the activity of DNA polymerase [382, 383] and therefore reduces the efficiency of PCR.

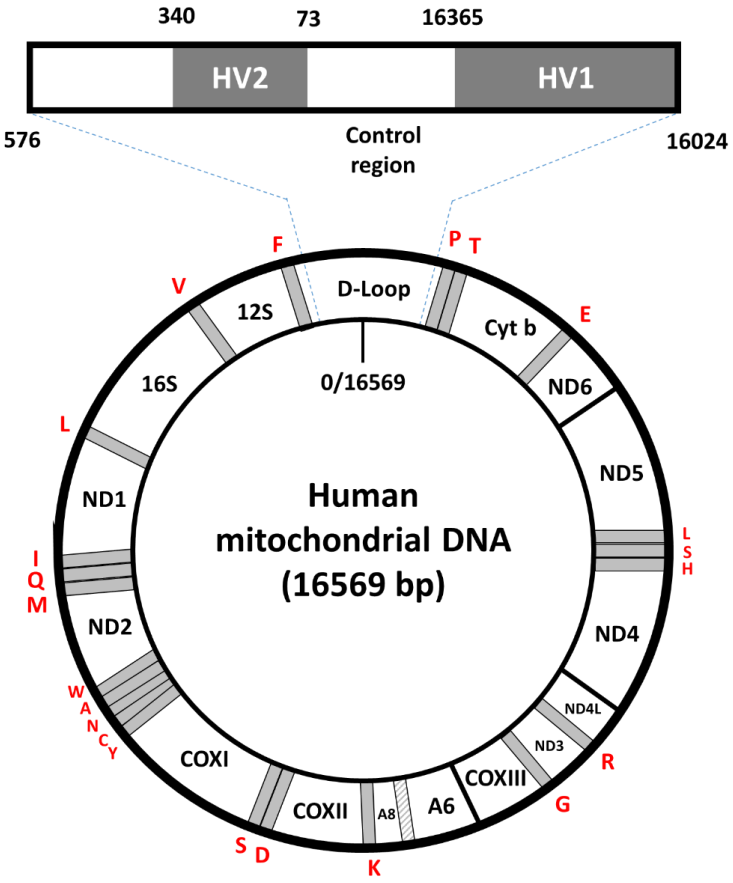


Figure 5-2 Schematic diagram of mtDNA and D-loop

The bold outer circle is representative of the heavy strand and the bold inner circle the light strand. Letters around the outside perimeter indicate cognate amino acids of the tRNA genes. The abbreviations are representative of the regions encoding the mRNA or rRNA.

5.1.3 Dating a hair by RNA

5.1.3.1 Determining the age of biological materials by measurements of RNA degradation

DNA analysis may provide a link between a suspect or a victim and the evidence at crime scenes and crucially affects the direction and even the results of criminal investigation. In many scenarios, the information provided by DNA analysis is sufficient for the conviction of the suspect. However, sometimes it is important to know the time when the biological evidence was deposited. Traditional DNA analysis

cannot provide such temporal information. Several studies have been presented to evaluate the time since deposition of a biological material. Most of these studies focus on bloodstains [384]. The techniques mostly are based on changes of the haemoglobin in red blood cells due to the degradation of blood proteins on exposure to air, measured by high performance liquid chromatography and spectroscopy [385, 386]. However, none of these methods have been widely accepted due to the variations resulting from environmental conditions.

Recently, several studies have focused on RNA extracted from the nucleus of white cells alternatively for dating the age of bloodstains. Expression of mRNA has been quantified in different tissues for identification and treatment of diseases [387, 388] or to monitor the effect of post medical treatment in medical science [389]. Scientists also studied the usage of mRNA quantification in the forensic field, for example, the identification of body fluids [154, 390]. Inoue *et al.* profiled the post-mortem degradation of house-keeping mRNAs, such as *GAPDH* and *ß-actin*, in a dead rat body by real-time PCR to check the relation between the mRNA decreasing level and post-mortem interval [391]. They found that the degradation rate of interleukin-1ß mRNA was higher than that of *GAPDH* in the lung of rats. Anderson *et al.* investigated the ratio of Cq values of *18S* rRNA and *ß-actin* mRNA from bloodstains over 150 days and found that the relative ratio of *18S* rRNA to *ß-actin* mRNA increased over time [177]. In theory, *ß-actin* is expected to degrade faster because its lack of protective protein complex which can shield it from degradation through environmental impacts, such as ubiquitous ribonuclease enzymes. In contrast, the rRNA molecule is combined with proteins to form the ribosomal complex structure, which protects it from being degraded by the ribonuclease enzymes [392]. Hence, rRNA is expected to have greater stability than mRNA. The results from Anderson *et*

al. provided support for the hypothesis that there are differences in *ex vivo* RNA decay rates between *18S* rRNA and *ß-actin* mRNA and confirmed that *ß-actin* mRNA is less stable and decays more rapidly than *18S rRNA* in *ex vivo* bloodstains. Anderson *et al.* also suggested that there is a great potential for applying this method to other body fluids or tissues as the targeted RNA markers are expressed in all types of cells. As both *18S* and *ß-actin* are considered as ‘‘housekeeping genes’’ which are expressed in all cell types at relatively high levels, these RNA products are likely to be found in all biological materials. Hence, the method of dating biological samples used by Anderson *et al.* is also possible to be applied on hair samples.

Compared to bloodstains, the sample size of hair evidence found at the crime scene is smaller. Hence much less genetic material could be collected in hair evidence. In addition, the degradation of hair is more prone to being influenced by environmental factors. Both factors cause it more difficult to date hair samples. However, it is still worth of research since hair is often present at crimes scenes. It is of interest to study and attempt to evaluate the possibility of dating the hair samples via measurements of RNA degradation.

5.1.3.2 The potential source of RNA in hairs

Hair is histologically divided into two different parts: a living cellular region and a dead acellular region. The living cellular region includes the hair root and shaft under the skin while the acellular region contains the hair shaft above the skin. Although Takumi Tochio et *al.* reported that the hair shafts above the skin preserve trace amounts of mRNAs (probably remnants expressed during hair differentiation) and are usable for microarray analysis [393], the amount of mRNA extracted from the hair

shaft may not be sufficient for further forensic analysis. Concerning the hairs collected at crime scenes, only a small quantity of genetic material can be extracted from the hair shaft and the genetic material is usually seriously degraded since the circumstances of the crime scene are usually critical. Besides, most studies on gene expression of the hair focus on the cellular region, and hair roots are considered as the ideal source of mRNA for genetic testing [394]. The cellular region of hair provides better quality and larger amounts of genetic material for experimental analysis. Hence, hair root is a better source than hair shaft for analysis.

5.1.3.3 Selecting plucked hairs as analysis materials

Most of the hairs found at crime scenes are either naturally shed hairs or plucked hairs resulted from physical contact or struggle. In this study, plucked hairs, instead of naturally shed hairs, were chosen as analysis materials for the following reasons. First of all, naturally shed hairs are telogen hairs which contain no adhering hair cells. As a consequence, they contain less DNA and RNA [363]. Secondly, the DNA quantity of these telogen hairs is usually highly degraded [360]. Genetic material in telogen hairs decays rapidly after shed from human body. The degradation rate accelerates due to the influence of the external environment such as temperature and humidity. Hence, it may take more time to analyse the shed hairs without satisfied results. Moreover, reproducibility is another problem for analysis with shed hairs because of the low quantity of genetic material contained in the shed hairs. All the above reasons make the telogen hair a bad choice for hair analysis.

Comparatively, plucked hairs may contain not only hair roots but also hair follicles which contain large amounts of genetic material. Therefore, plucked hairs are

a better choice than shed hairs for forensic analysis. Hampson *et al.*, instead of using telogen hairs, also chose plucked hairs as the experimental materials in their study to estimate the aging of forensic samples [192]. Likewise, under the consideration of experimental efficiency, plucked hairs were used as hair samples to obtain more cells from hairs in this study.

In addition to the consideration of experimental efficiency, there is also a practical value of dating the time since the hair was plucked. Shed hairs found at crime scenes may have been left there for a long time before the occurrence of the crime and are irrelevant to the suspects or victims while plucked hairs collected at crime scenes are usually related to either the suspects or victims and often discovered in violent cases, especially murder, robbery and sex assault cases. Identification of the plucked hairs can provide corroborating link to a suspect and can be critical evidence to criminal investigation. Sometimes, it is also important to determine the time when the plucked hair was deposited at a crime scene, which provides temporal information related to the occurrence of the crime event and the presence of suspects. From this viewpoint of criminal investigation, the practical value of dating plucked hairs might be higher than shed hairs in forensic analysis.

5.2 Materials and methods

The flowchart of this study is shown as Figure 5-3. Each step will be addressed in sequence.

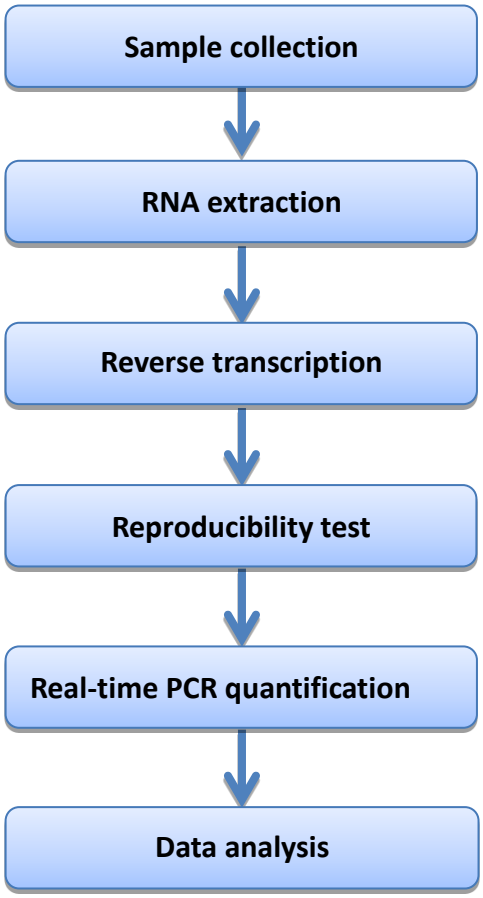


Figure 5-3 Flowchart of this project

5.2.1 Sample collection

Hair samples were collected from a total of 8 consenting Taiwanese volunteers including 4 females (28 to 57 years old) and 4 males (25 to 36 years old). Hairs were plucked from each volunteer weekly for each time point (day 0, 7, 14, 21, 28, 35, 42, 49, and 56). The procedure was approved by Institutional Review Board (IRB) of

Central Police University in Taiwan. Plucked hairs were examined microscopically to ensure the presence of the hair follicles. Later, the hairs were packaged with A4-sized paper and left to age at room temperature. RNA extraction was conducted all together after the hairs were collected at all time points.

The last 0.8 cm of each hair shaft, inclusive of the adhering follicle, was cut and stored in a sterile 1.5ml Eppendorf tube. Each tube contained 3 hair roots and one tube constituted one sample for each RNA extraction of each donor. A total of 6 hairs (two samples) were collected from each person for each time point, ensuring there were duplicate samples for each time frame. Hence, there were totally 144 samples (8 donors × 9 time points × 2 samples). The hairs were washed by 99% ethanol to remove residual substances on the hair surface as the residual ethanol might contaminate the sample and affect the quantification of RNA. The hairs were then incubated at room temperature for ethanol removal before RNA extraction to prevent contamination from chemicals and/or biological materials that might affect the reaction of the experiment.

5.2.2 RNA and cDNA preparation

RNA was extracted from the hair samples by TRI REAGENT kit (Molecular Research Centre, Inc., Cat. No: TR118) with the presence of RNase inhibitor (Promega Corporation, Cat. No: N2111). For each sample, 300µl TRI Reagent was added into each tube, and then the tube was immersed in liquid nitrogen to freeze the reagent with hairs. After the reagent was frozen, the tube was removed from liquid nitrogen and kept at room temperature. After thawing out, the reagent was immersed in liquid nitrogen to freeze again. This step was repeated for three times to break and

homogenize the cell by keeping the change of temperatures. Then 30µl bromochloropropane (BCP) was added to partition the RNA into the aqueous phase. The sample was incubated at room temperature for 5 minutes, vortexed for 15 seconds, and then cooled down to 4˚C for 15 minutes in sequence. The sample was then centrifuged at 12000g for 15 minutes at 4˚C. The supernatant (RNA layer) was transferred to another tube afterward. The liquid was maintained at -80˚C for 2 hours after 150µl isopropanol and 0.5 polyacryl carrier (Molecular Research Center Inc., Cat. No: PC152) were added. Then the solution was centrifuged at 12000g for 30 minutes at 4˚C. The RNA pellet was washed by 300µl 75% ethanol. The solution was then centrifuged at 7500g at 4˚C for 5 minutes and then air-dried for 15 minutes. Finally, DEPC water was added and RNA was preserved at -80˚C.

DNA contamination may occur during RNA extraction. To avoid genomic DNA carryover, the RNA extraction product was treated with DNase. Rnase-free DNase (Promega Corporation, Cat. No: M610A) was used for DNA decontamination. The decontaminating reagents include (1) 10X Reaction Buffer (M198A): 400mM Tris-HCl (pH8.0), 100mM $MgSO_4$ and 10mM $CaCl_2$. (2) Enzyme Storage Buffer RQ1 DNase: Supplied in 10mM HEPES (pH7.5), 50% glycerol (v/v), 10mM $CaCl_2$ and 10mM $MgCl_2$. (3) Stop Solution (M199A): 20mM EGTA (pH8.0). Before performing the reaction of reverse transcription, 10µl of 1X Reaction Buffer with RQ1 RNase-Free DNase (1U) was added to the RNA sample. The sample was incubated at 37˚C for 30 minutes and then 1µl RQ1 DNase Stop solution was added to stop the reaction. The solution was then stored at 65˚C for 10 minutes for DNase deactivation. The final solution (11µl) was then ready to be applied for RNA reverse transcription.

After RNA extraction, cDNA was synthesized with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Cat. No: 4362214, see Section 2.1.3). Reverse transcription was carried out in a thermal cycler (model 9700, Applied Biosystems).

5.2.3 Marker selection

As mentioned in Section 5.1.3, the differences in *ex vivo* RNA decay rates between *18S* rRNA and *ß-actin* mRNA had been evaluated by Anderson et al. and confirmed that *ß-actin* mRNA is less stable and decays more rapidly than *18S* rRNA in *ex vivo* bloodstains. The *18S rRNA* has been introduced in Section 3.2.3. As to *ß-actin* mRNA, it is one of six actin isoforms and actin is essential to cytoskeleton and many fundamental cellular processes, which makes it very important to eukaryotic cells [395]. It is found in all eukaryotic cells except nematode sperms which contain no filamentous (F-actin) [396]. As considered as housekeeping genes, both *18S* rRNA and *ß-actin* mRNA are expressed in all types of cells, including the cells of the hair follicle, at relatively high and constant levels [177]. Besides, highly specific TaqMan® probes for both markers are commercially available, so primer design and pre-test for the reaction are not required. Both markers can be tested at the same time by labelling them with different fluorescent dyes. Thus, *18S* rRNA and *ß-actin* mRNA were selected as the markers for the measurement of RNA degradation.

5.2.4 Real-time PCR quantification

To observe the decaying rate of different RNAs, relative quantification by real-time PCR was used to measure the change of *18S* rRNA and *ß-actin* mRNA extracted from the hairs plucked at different time points. The TaqMan® Universal PCR Master

Mix (Applied Biosystems™, Cat. No: 4304437) was used for this reaction. It contains AmpliTaq Gold® DNA polymerase, Uracil-N glycosylase, dNTPs with dUTP, ROX™ Passive Reference, and other optimized buffer components. The ROX™ dye is a passive internal reference for normalizing the signal. Normalization is important as there might be changes in concentration or volume of the reaction which result from evaporation or pipetting. Besides, the TaqMan® ß-actin Detection Reagents (containing *ß-actin* primer pair and probe with FAM dye; Applied Biosystems™, Cat. No: 401846) and the TaqMan® Ribosomal RNA Control Reagents (containing 18S rRNA primer pair and probe with VIC™ dye; Applied Biosystems™, Cat. No: 4308329) were also used in this study. The preparation of the reagent mixture is listed in Table 5-1. Four microlitres of each cDNA was added to the PCR master mix to make a final reaction volume of 25µl. Duplicate quantification by real-time PCR was performed for each cDNA.

Table 5-1 Reagents for Real-Time PCR quantification reaction

Reagent	1 reaction	Final concentration
2x TaqMan mixture	12.5µl	1X
ß-actin Forward primer (3µM)	2.5µl	300 nM
ß-actin Reverse primer (3µM)	2.5µl	300 nM
ß-actin probe with FAM dye (2µM)	2.5µl	200 nM
18S rRNA Forward primer (10µM)	0.125µl	50 nM
18S rRNA Reverse primer (10µM)	0.125µl	50 nM
18S rRNA probe with VIC™ dye (40µM)	0.125µl	200 nM
Water	0.625µl	
Template	4µl	
Total	25µl	

The real-time PCR was carried out using Applied Biosystems™ 7900HT Fast Real-Time PCR System (Applied Biosystems™) following the manufacturer's instructions. The thermal program is listed as Table 5-2. The data were analysed using SDS software (version 2.2.2, Applied Biosystems™).

Table 5-2 Thermal program for real-time PCR quantification reaction

Temperature	95°C	95°C	60°C	4°C
Time	10 min	15 sec	1 min	∞
Cycles	1	45		

In order to reflect the quantity of the target within the real-time PCR accurately, measurement at the optimized point is critical (see Section 2.3). Parameters, including baseline and threshold, should be accessed carefully. The baseline is set from cycles 3 to 15 by default and the default value assigned for the threshold is set as 10 times the standard deviation above the mean baseline fluorescence by the software (Figure 5-4). Although the threshold value can be adjusted manually afterward to get better precision and sensitivity, in most cases for a given assay with the same primer and probe set, using the same threshold and baseline settings is suggested by the manufacturer as it will improve the precision and make the data more comparable from plate to plate [397]. Hence, in this study, a fixed value (0.05) was set as the threshold for each plate with the reference of the optimized value automatically set by the SDS software.

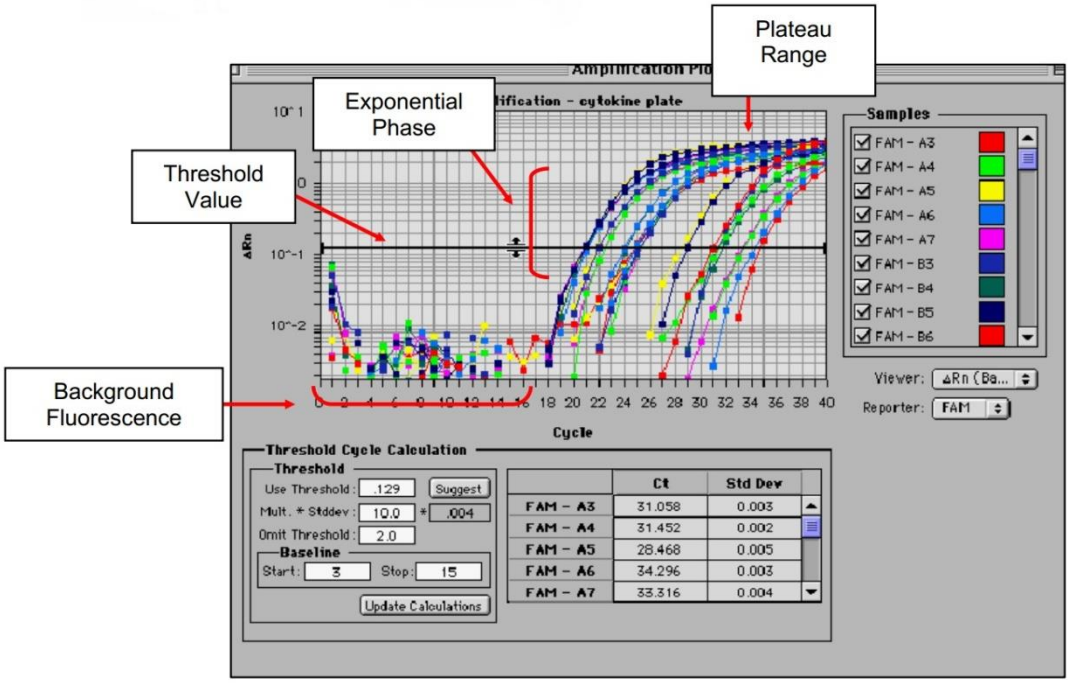


Figure 5-4 A good threshold setting for the real-time PCR

The points during the exponential phase of amplification are suggested to be the most accurate for a measurement of the amplicon. Thus, the thresholds were set in the range of the exponential phase of the PCR. The software can set the baseline and threshold automatically. The default setting for the baseline is from cycle 3 to 15. Then the default threshold is calculated as 10 × (standard deviation of baseline) above the mean value of the baseline. However, the setting may need to be adjusted slightly due to changes in the reaction medium which creates a fluctuating background signal. Adopted from "Data Analysis on the ABI PRISM® 7700 Sequence Detection System: Setting Baselines and Thresholds".

5.2.5 Preliminary test

Experimental errors (such as pipetting errors, which cannot be avoided completely) may lead to significant errors in real-time PCR, especially when the amount of RNA extracted from hair samples is low. Although the TaqMan® Universal PCR Master Mix contains ROX™ dye which is used to normalize the signal, the efficiency test was still conducted to confirm its robustness in the preliminary test. The human control DNA from the TaqMan® ß-actin Detection Reagents kit (Applied Biosystems, Cat. No: 401846) was used as the control sample for the preliminary test. The preparation of the master mix was carried out in two ways (Figure 5-5). The first

way was to mix all required reagents for real-time PCR with control DNA together and then aliquot the mixtures to each reaction tube. The other way was to mix the reagents and aliquot them to each tube in advance and then add the control DNA to each tube respectively. The precision of the method, including the operation of the instrument and the preparation of mixtures, was evaluated by calculating the average and standard deviation.

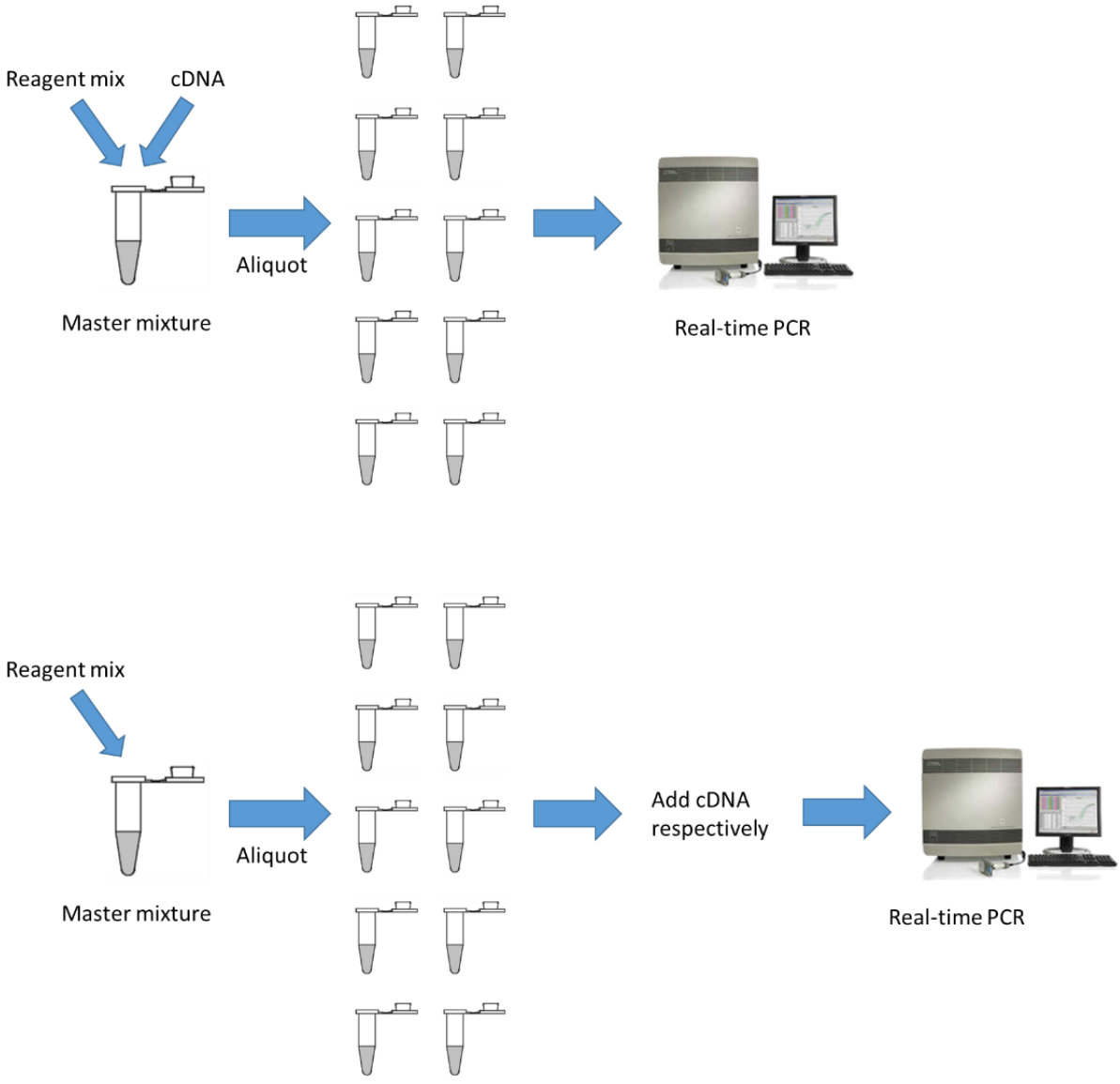


Figure 5-5 Schematic diagram depicting the difference of the methods

5.2.6 Data analysis

Quantification cycle (Cq) values of *18S* rRNA and *ß-actin* at each time point for each donor were exported from the SDS software and the difference of the Cq values between *18S* rRNA and *ß-actin* mRNA (dCq) at each time point was then calculated ($dCq = Cq_{ß\text{-}actin} - Cq_{18S\ rRNA}$) for further analysis. In this study, the difference of the Cq value between *18S* rRNA and *ß-actin* mRNA was calculated for analysis instead of comparing the Cq value directly. Theoretically, the influence of sampling issues can be neutralized in this way. Variation of each time point was analysed by comparing the relative standard deviation. Regression analyses, including linear and quadratic regression, were used to examine the correlation between the dCq value and the time when the hair was plucked from each donor. Finally, the equation of correlation was explored for an approximate estimation of the time since the hair was plucked.

5.3 Result

5.3.1 Preliminary test

Two different methods about reagent preparation were tested and compared to examine the difference. The result is shown in the box plot (Figure 5-6). The first method was to mix the reagents and control DNA in advance and then aliquot to each reaction tube (n=10). The average dCq value between *18S* rRNA and *ß-actin* mRNA ($Cq_{ß\text{-}actin} - Cq_{18S}$) was 4.74 and the standard deviation of the dCq values was only 0.21 (4.3%). The other method was to mix the reagents and aliquot them to each reaction tube (n=9) in advance and then the control DNA was added to each reaction tube. The average dCq value between *18S* rRNA and *ß-actin* mRNA was 4.65 and the standard deviation of the dCq values was 0.18 (3.9%). For the statistical hypothesis testing at a

95% confidence level (α=0.05), there is no significant difference between the two groups of dCq values from the two methods (t=0.9997 < $t_{17,0.025}$=2.1098). This indicated that the pipetting difference resulting from mixture preparation for real-time PCR can be neutralised by the internal control.

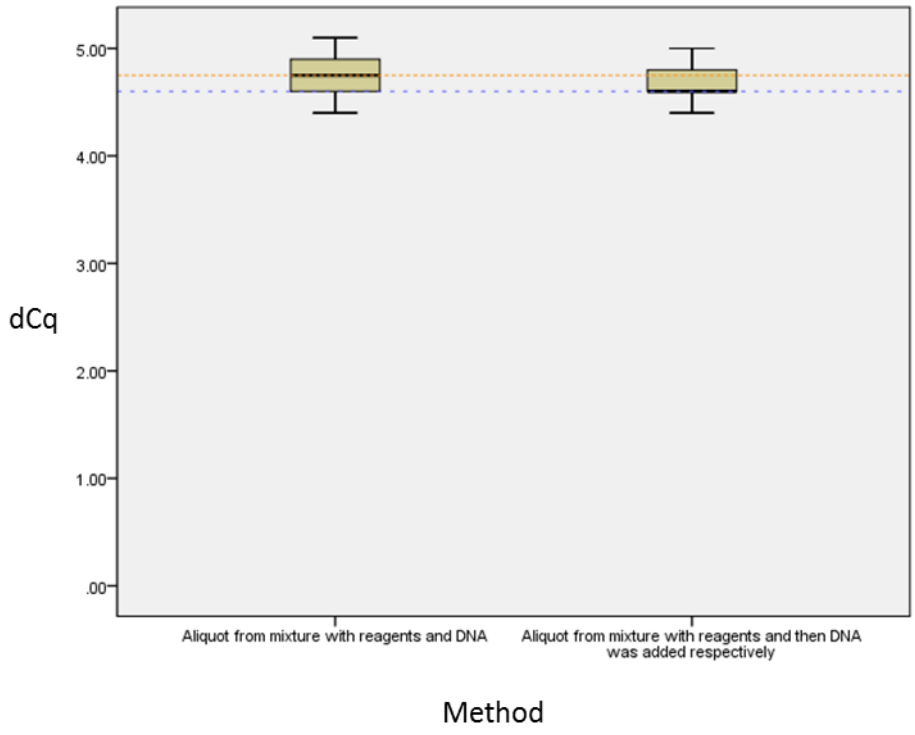


Figure 5-6 Box plot showing the difference of Cq (dCq) between 18S rRNA and ß-actin mRNA using real-time PCR

The box plot depicts the distribution of the result. The bottom and top of the box are the first and the third quartiles respectively, and the line inside the box is the second quartile of dCq of each method. The ends of the whisker represent the minimum and maximum of the dCq value.

5.3.2 Cq values of 18S rRNA and ß-actin mRNA

The average Cq values for *18S* rRNA and *ß-actin* mRNA at each time point are shown in Figure 5-7. Regarding the Cq values, no time-wise trend was found in both markers. This is because the Cq value is directly relative to the amount of template cDNA used for the real-time PCR. The template cDNA used in this study for real-time

PCR was reverse transcribed from total RNA which was extracted from hair samples directly without quantification in advance. Thus, the amounts of cDNA used for real-time PCR varied in each test. As a consequence, no correlation between the Cq value and time for both markers was expected and the result supported this. Nevertheless, at least it was proved that both targets were successfully amplified from hair samples in the real-time PCR reaction. In this study, the difference of the Cq value between *18S* rRNA and *ß-actin* mRNA was calculated for analysis instead of comparing the Cq value directly. Theoretically, the influence of sampling issues could be neutralized in this way.

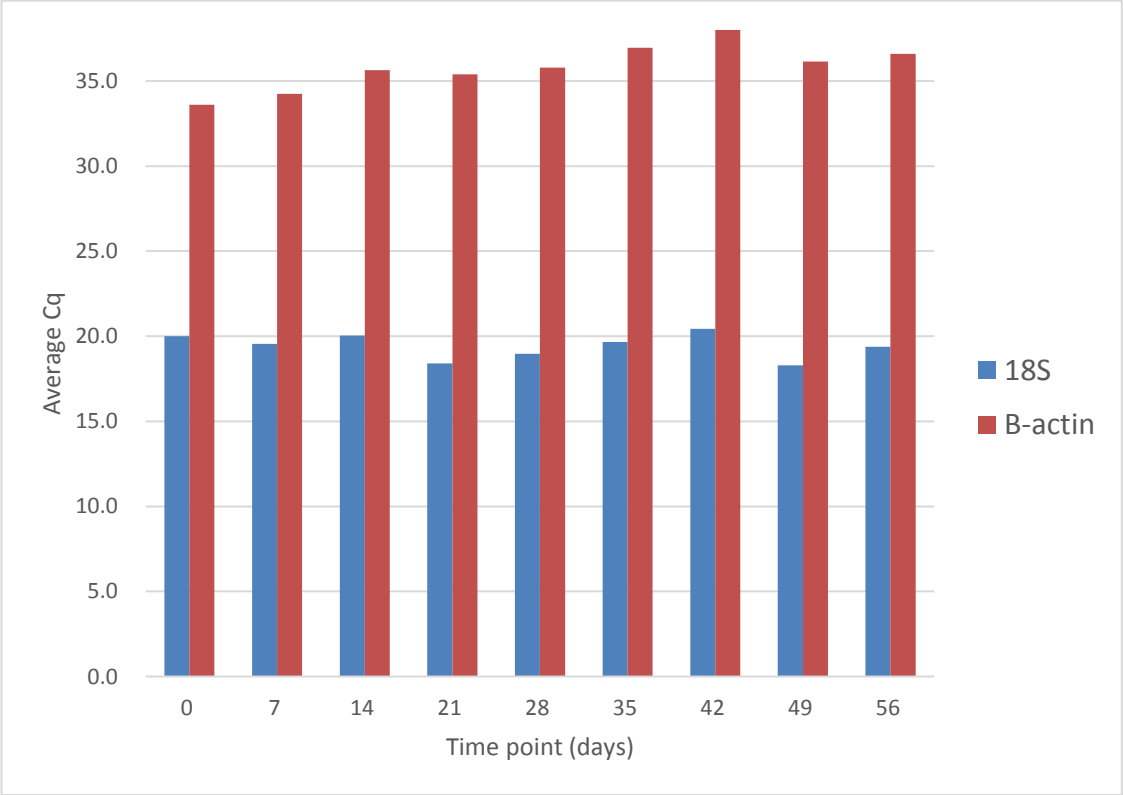


Figure 5-7 The Cq values for 18S rRNA and ß-actin mRNA at each time point

5.3.3 The time-wise trend of dCq of each donor

With the assumption that *ß-actin* mRNA decomposes faster than *18S* rRNA (see Section 5.1.3.1), the dCq value should increase over time after hairs were plucked. The change of the average dCq value of each donor at different time points was analysed to investigate if there is a time-wise trend shown between the time and the average dCq value. For each person, the average of the dCq value of each time point (Appendix 8) and the correlation coefficients (linear and quadratic relationship) were calculated respectively based on the time points and the average dCq values (Figure 5-8).

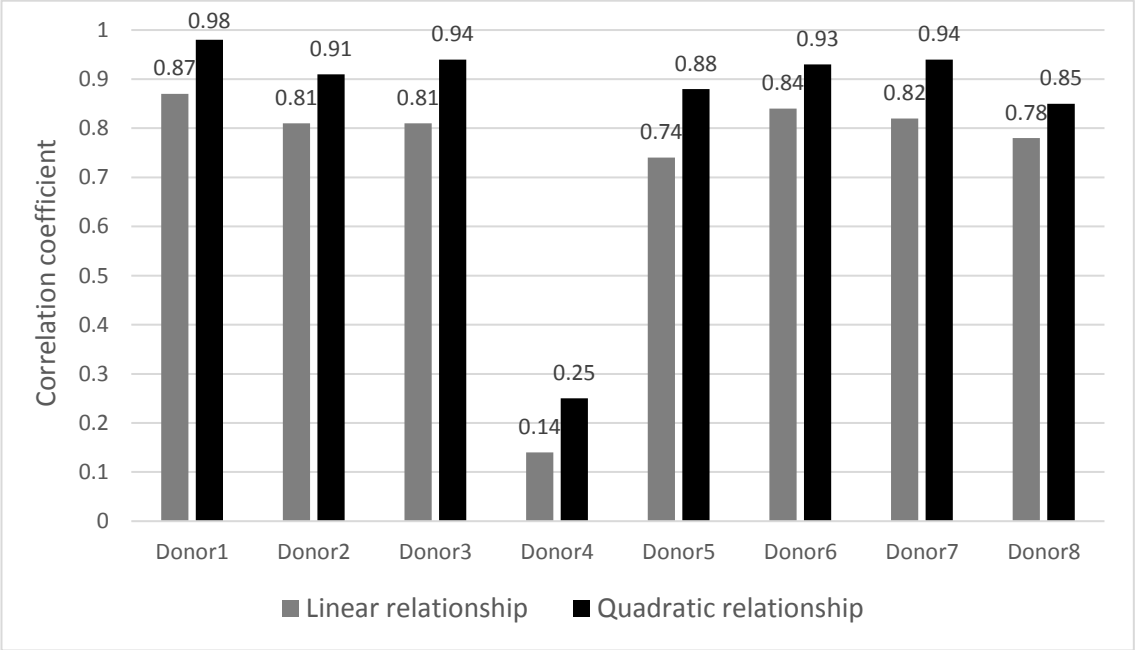


Figure 5-8 The comparison of correlation coefficients (linear and quadratic relationship) for the correlation between the time and the average dCq value for each donor

As shown in Figure 5-8, for all of the donors, the values of correlation coefficient in a quadratic relationship were higher than that in a linear relationship. This suggested that a quadratic relationship was better to represent the correlation

between the time since the hair was plucked and the average dCq value. Besides, according to the guide reported by Evans [398], the strength of the correlation could be described by the absolute value of correlation coefficient (Table 5-3). The values ranged from 0.74 to 0.87 for linear relationship and from 0.85 to 0.98 for quadratic relationship for all donors except donor 4. Based on the guide shown in Table 5-3, all correlation coefficients were considered very strong positive correlation except that of donor 4.

The result of donor 4 was obviously different from the other donors. The values of correlation coefficient of donor 4 in both linear relationship and quadratic relationship were extremely low (0.14 for the linear relationship and 0.25 for the quadratic relationship), which showed a very weak correlation. A further enquiry about the hair condition revealed that the donor had her hair permed and dyed during the time of sample collection. Several hair dye components, such as carcinogenic 4-nitro-2-aminophenol and 5-nito-2-aminophenol, may induce DNA cleavage frequently [399]. Several studies also have revealed that hair dyeing makes DNA damage in human lymphocytes and raises the risk of leukemia and bladder cancer [400, 401]. The same factors may also induce the cleavage of RNA [402]. During the process of hair dyeing, the dye chemicals are applied on the surface of the hair and the scalp for a while and might directly contact the hair follicles, which increases the possibility of damage to nucleic acids. Although the level of damage cannot be evaluated easily, it is possible that hair dyeing may account for the low correlation between the time since the hair was plucked and the average dCq value shown in donor 4.

Table 5-3 The description for the value of the correlation coefficient based on Evans's guide (1996)

The value of correlation coefficient	Description
0-0.19	Very weak
0.20-0.39	Weak
0.40-0.59	Moderate
0.60-0.79	Strong
0.80-1	Very strong

5.3.4 Variation of each time point

The average, standard deviation, and relative standard deviation of the average dCq values for each time point were calculated to estimate the variation between different time points (Table 5-4). The standard deviation ranged from 1.2 (day 28) to 2.8 (day 7). Besides, the relative standard deviation of each time point revealed a decrease tendency as time went by and the standard deviation showed roughly the same tendency. A larger standard deviation was found at the initial time points from day 0 to day 21, which represented that the data points were spread out over a wider range. After day 21 (from day 21 to day 56 in our study), the standard deviation decreased and maintained in a relatively low level. This phenomenon might result from the following reason. After being plucked from the volunteers, hairs were microscopically examined to ensure the presence of hair root. However, as the hair turns from one phase into the next gradually without a clear cutting point, hairs on the scalp might be in any of the three phases. As a consequence, although most of the hairs (85% to 95%) are anagen hairs (see Section 5.1.1), the collected hairs might possibly also include a few telogen hairs. It is supposed that the nucleic acids start to decay after the catagen or anagen hairs are plucked while the nucleic acids in telogen hairs have already started to decay before the hairs are plucked. Hence, comparing

hairs of the three phases plucked at the same time, the degradation in the telogen hairs is faster than that in the hairs of the other two phases. The effect of earlier starting degradation in the telogen hairs may result in the spreading result at the initial time points, causing a larger standard deviation in the beginning points. This effect becomes less effective, resulting in small deviation of the result at the later time points.

Furthermore, it was found that the increase of the average dCq value between *18S* rRNA and *ß-actin* mRNA tapered off gradually after 21 days. Hampson *et al.* represented that there was a noticeable decrease in the *18S* rRNA relative expression in the older samples [192]. In other words, the degradation of *18S* rRNA became faster along with the time, reducing the average dCq values between *18S* rRNA and *ß-actin* mRNA after 21 days. This finding was also supported by our study.

Table 5-4 The average, standard deviation, and relative standard deviation of dCq for each time point

Time point	Average of dCq	Standard deviation of dCq	Relative standard deviation
0	13.4	2.1	15.82%
7	14.7	2.8	18.78%
14	15.6	1.9	12.12%
21	17.0	1.8	10.65%
28	16.8	1.2	7.23%
35	17.3	1.5	8.76%
42	17.6	1.3	7.50%
49	17.8	2.0	11.06%
56	17.2	1.3	7.60%

5.3.5 The correlation between the time and the average dCq value

Comparing the average dCq values of each time point (Table 5-4), there was a noticeable increase from day 0 to day 21 (from 13.4 to 17.0). After day 21, the increase

of the average dCq value was getting slow within a range from 16.8 (day 28) to 17.8 (day 49). This indicated a positive correlation between the average dCq value and the time since the hair was plucked. Besides, the time-wise trend was significant at the initial time points and tapered off at the following time points.

A regression analysis was performed with the average dCq values and the time points to evaluate the correlation statistically. Both linear and quadratic regression analyses were performed (Figure 5-9 and Figure 5-10) and the correlation coefficients for both regression analyses were also calculated respectively to evaluate the correlation and to determine the best model for the regression.

In the linear regression, the correlation coefficient was 0.88 (r^2=0.78, Figure 5-9), which represented a moderate positive correlation while the correlation coefficient was 0.99 (r^2=0.98, Figure 5-10) in the quadratic regression. The mean squares of residual (residual sum of square divided by the degrees of freedom) for linear and quadratic regression were 0.58 and 0.08 respectively. In terms of the correlation coefficient, the curve of the quadratic regression was better than the linear regression to describe the correlation between the average dCq value and the time since the hair was plucked. With the linear regression, the time since the hair was plucked (TSP) could be approximately evaluated by the average dCq value with the regression equation (Figure 5-9):

$$TSP = 0.069 \times dCq + 14.445$$

With the quadratic regression for all time points, the time since the hair was plucked could be approximated by the average dCq value using a second-order polynomial (Figure 5-10):

$$TSP = -0.002 \times dCq^2 + 0.193 \times dCq + 13.436$$

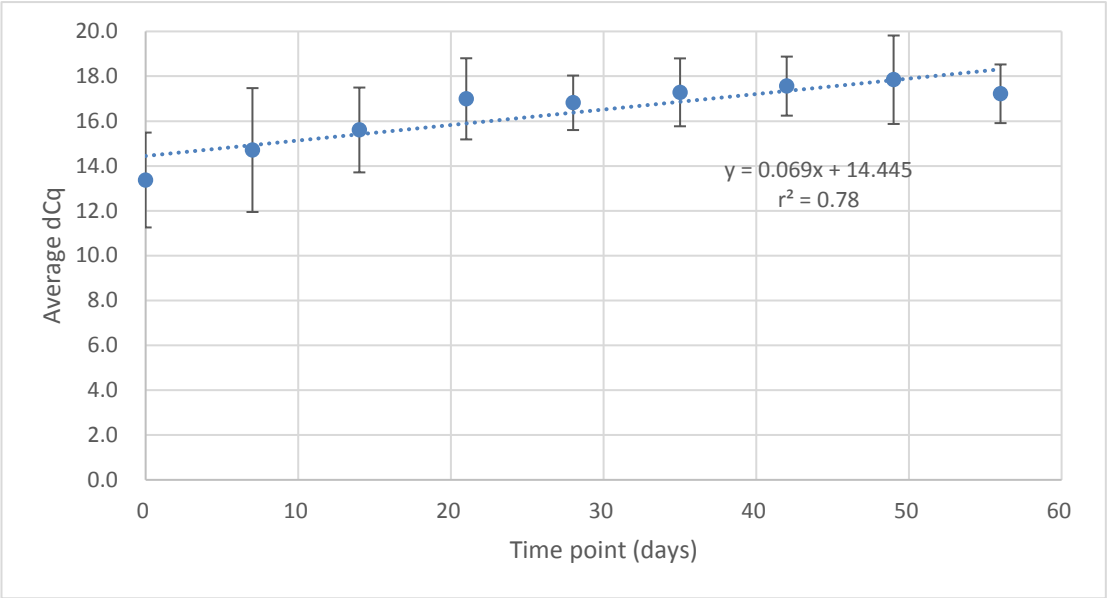


Figure 5-9 The linear regression analysis of the time points (day 0 to day 56) and the average dCq

The dotted line represents the regression curve and the equation for the regression curve is shown below the curve. The range of the error bar represents the standard deviation of each data point.

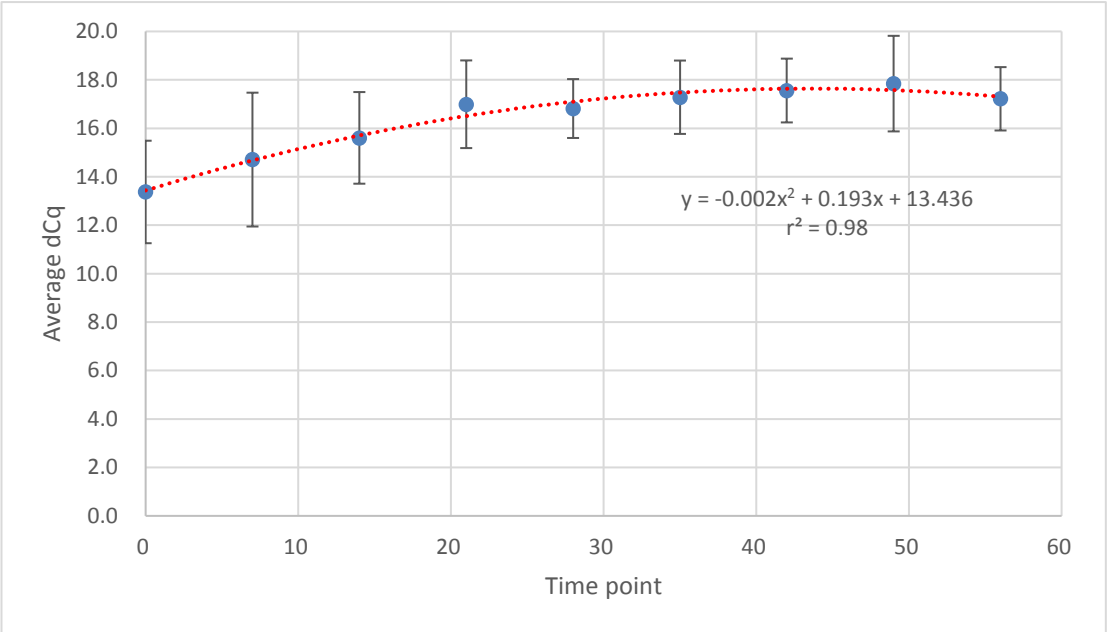


Figure 5-10 The quadratic regression analysis of the time point and the average dCq

The dotted line represents the regression curve and the equation for the regression curve is shown below the curve. The range of the error bar represents the standard deviation of each data point.

Nevertheless, if only data at the time points from day 0 to day 21 were considered, the correlation coefficient was 0.997 (r^2=0.99, Figure 5-11) for a linear regression, which represented a strong positive linear correlation. After day 21, the average dCq values kept in a relatively constant level (the average dCq values between day 21 and day 56 was 17.3 and the standard deviation was 0.38). The strong positive linear correlation was replaced with a plateau effect (Figure 5-12). This implied that there is a positive correlation between the average dCq value and time within the first 21 days. This equation could be applied to estimate the age of the plucked hair. The time after the hair was plucked could also be approximated with a linear polynomial (Figure 5-11):

$$TSP = 0.168 \times dCq + 13.406$$

Comparatively, the linear polynomial could be better to predict the time since the hair was plucked within 21 days according to our study. After 21 days (from day 21 to day 56), the time-wise trend was not significant and the average dCq values kept in a relatively constant level (Figure 5-12).

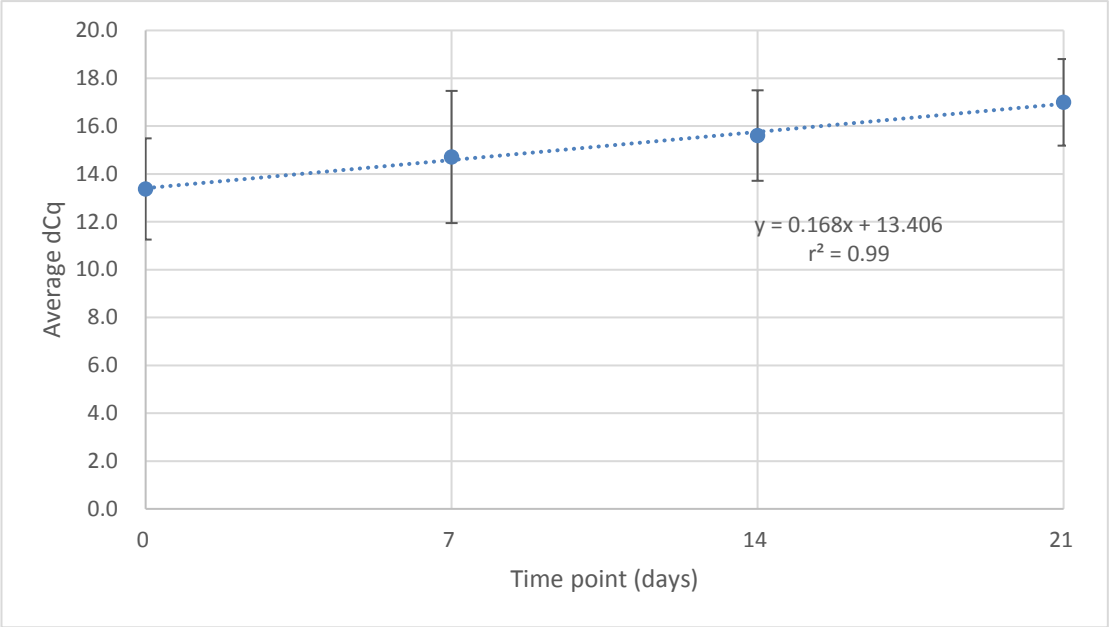


Figure 5-11 The linear regression analysis of the time points (day 0 to day 21) and the average dCq

The dotted line represents the regression curve and the equation for the regression curve is shown below the curve. The range of each data point represents the standard deviation for each data point.

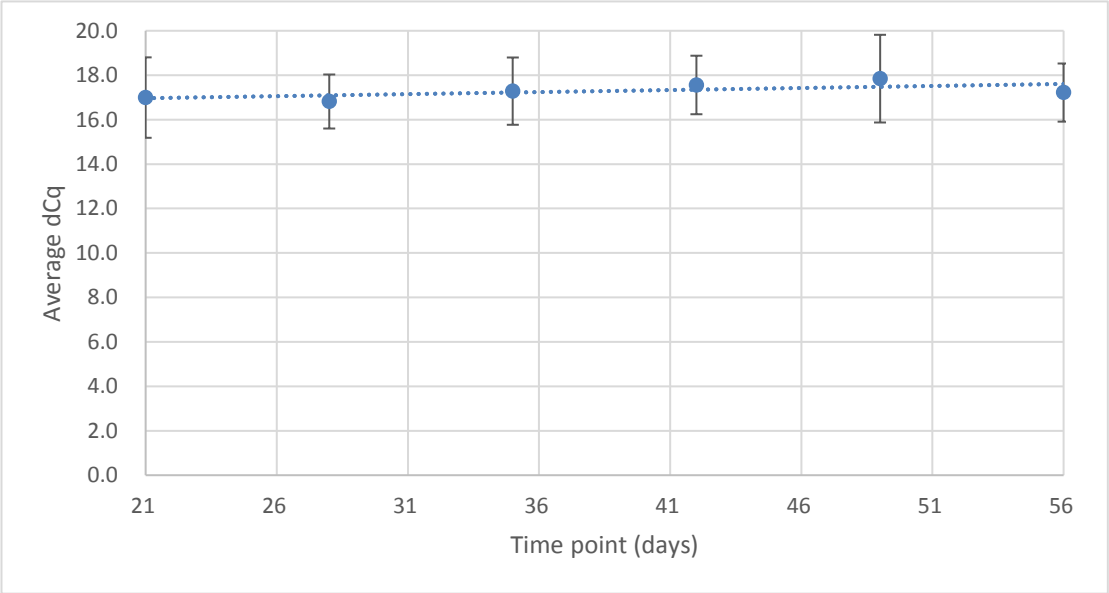


Figure 5-12 The dCq values at different time points (day 21 to day 56) and dCq

The average of the dCq between day 21 and day 56 is 17.3 and the standard deviation is 0.38.

5.4 Discussion

The technology to determine the time since deposition of biological materials collected at the crime scene helps investigators to establish the time of commission of criminal offences. Several studies have been proposed for estimating the age of bloodstains since it is the most common biological evidence found at the crime scene. Hair samples are also usually found at the crime scene but often ignored by investigators. In this study, we demonstrated the possibility of age estimation of plucked hairs from examining the relative degradation between two different RNA species (*18S* rRNA and *ß-actin* mRNA). The influence by different amounts of template cDNA used for real-time PCR reaction could be neutralized by calculating the dCq value of *18S* rRNA and *ß-actin* mRNA since it reflected the relative quantification. It has also showed that using an internal control could avoid pipetting difference resulted from the progress of the mixture preparation for real-time PCR in our preliminary test.

Both linear and quadratic regression analyses were performed to examine the correlation between the average dCq value and the time since the hair was plucked. The results showed a time-wise trend between the two factors. Based on all time points, a linear polynomial (TSP = 0.069 × dCq + 14.445, r^2=0.78) and a second-order polynomial (TSP = -0.002 × dCq^2 + 0.193 × dCq + 13.436, r^2=0.98) were obtained from linear and quadratic regressions, respectively. It was found that the quadratic regression is better than the linear regression in describing the correlation between the average dCq value and the time since the hair was plucked according to the correlation coefficients and comparison between the mean squares of residual (0.5 and 0.08 respectively). Further analysis showed that the correlation between the average dCq value and the time could be divided into two periods: day 0 to day 21 as

one period and day 21 to day 56 as the other. Comparing with the quadratic relationship between the dCq value and the time since the hair was plucked, the linear correlation is more reliable in describing the relationship between the average dCq value and the time points from day 0 to day 21. The average dCq value increased over time in a linear manner with a linear equation: TSP = 0.168 × dCq + 13.406 (r^2=0.99). After 21 days, the time-wise trend for the change of the average dCq value became less significant and a plateau effect was observed. This indicated that the relative degradation rate between *18S* rRNA and *ß-actin* mRNA was significant in the first 21 days. Although this equation might not be applied to predict the time since the hair was plucked directly, it still suggests the possibility to estimate the time since the hair was plucked by this assay. After 21 days, the relative degradation rate became almost in the same level and was no longer useful for the estimation.

It is thought that the degradation of *18S* rRNA keeps a slow rate and the degradation of *ß-actin* mRNA is faster than that of *18S* rRNA [177]. However, Hampson *et al.* represented a noticeable decrease in the *18S* rRNA relative expression in older hair samples in their study, indicating that the degradation of 18S rRNA became faster after a certain time [192]. The degradation of *18S* rRNA increased later owing to the breakdown of the ribosomal complex, which is believed to be a structure to protect the rRNA from being attacked [177]. Without the protection of the ribosomal complex, the *18S* rRNA degraded faster and kept at a certain level. Different from *18S* rRNA, the degradation rate of *ß-actin* mRNA consistently kept at the same level. It can be concluded from the result that the average dCq value and the time kept in a linear correlation in the first 21 days. After 21 days, there was no linear correlation between the two factors as the dCq value roughly kept at the same level.

It was also observed that the average dCq value decreased slightly at the last time point (day 56). The quadratic equation for the correlation also indicated a slight falling tendency at this time point. Although it was impossible to examine the trend after 56 days as it was the last time point tested in this study, it was believed that the average dCq value should not carry on falling but will keep at a certain steady range without obvious change. This is because the degradation rate of *18S* rRNA and *ß-actin* mRNA should be approximately the same after the turning point (day 21). This means the lower dCq value shown in day 56 might be a deviation caused by other factors.

Another issue we noticed is that the standard deviation of the average dCq value for each time point was relatively larger comparing with the change of the average dCq value between two successive time points. The phenomenon of a larger standard deviation at a single time point might result from two reasons. One is the small population tested since there were only 16 samples each time (8 donors × 2 samples). A small amount of samples might not only result in a larger standard deviation, but also distort the tendency of correlation. The other reason attributes to the various uncontrolled environmental conditions such as room temperature and humidity. These environmental conditions could be factors affecting the degradation of nucleic acids and interfering with the correlation between the average dCq value and time. The deviation could be reduced by including more samples for each time point, as well as conducting the procedures in a more controlled environment.

Based on the result, a model that could approximate the time since the hair was plucked was established by measuring the difference between the expression level of *18S* rRNA and *ß-actin* mRNA within 21 days. Although the time frame tested in this study (day 0 to day 56) and the concluded effective time (day 0 to day 21) are limited, it is still useful for certain scenarios involving weekly scale of time window to

determine the presence or absence of the suspect or victim at the crime scene. The time since the deposition of the plucked hair could possibly link the suspect to a specific crime scene during a specific time range. Besides, several methods involving biology, anthropology, and archaeology have been applied on determination of the age of biological materials in different time frames [403]. Along with the method of dating the age of hairs since deposition used in this study, these methods could be joined collaboratively to estimate a reliable time of evidence since deposition.

The major problem inherent in most forensic dating methods, such as dating the hairs by RNA in this study, is that degradation of samples always does not involve solely a function of time. Many environmental factors may play a role in the rate of degradation of RNA. These variables make the estimation of the age of biological samples a difficult task. The interfering factors can be even more complicated in hair samples and result in larger variations in degradation. Although efforts have been made to control the environmental conditions in this study, including temperature, humidity, light, and air flow, it is impossible to keep all samples in the same condition.

The phase of the plucked hairs is another influence factor of degradation. The degradation rate of *18S* rRNA and *ß-actin* mRNA in the telogen hair may be different from that in the anagen or catagen hair. Hairs of all three phases exist on the scalp simultaneously and therefore the collected hairs may belong to three different phases, which would result in large variations of the dCq value in the beginning. It is suggested more samples (for example, using ten hairs, instead of three hairs, with follicles attached for RNA extraction at each time point for each donor) should be used to reduce the influence from the variations of sampling in different hair phases.

Chemicals used for hair dye or perm are another potential influence. The result of donor 4 in our study was significantly different from the other donors in that her result indicated no obvious correlation between the dCq value and time. One possible reason for this unusual result is due to the chemicals used in hair dye or perm as they might induce the cleavage of RNA and cause damage to nucleic acids. Therefore, this assay might not be applicable to dyed or permed hairs. As it is prevalent for people to have their hair dyed or permed, additional hair conditions, like dyed or permed hairs, should be noticed before estimating the age of the hair samples with this method.

The case of donor 4 also raises another methodological issue in sample collection. In our study, in order to reduce the discomfort of donors from hair plucking, the hairs for test were plucked periodically and then all samples were applied for the experiment at the same time. This however, will inevitably increase the possibility of other influence factors caused by the change of hair conditions during the time of hair collection. One way to avoid such interfering factors is to alter the method of hair collection. That is, to collect the hairs for test at once and then conduct the experiment with the hair samples periodically to explore the issue in dating hair samples.

To sum up, our study demonstrated the possibility of dating hairs via RNA. Nevertheless, the application made in this study is limited because of the small tested population (n=8), the short tested time frame (within 56 days), and requirements of the amount of evidence (3 hairs), specific hair samples (with attached follicular tag), and the controlled environmental variants such as temperature, humidity, and contamination. Besides, forensic samples found at the crime scene usually have been left in a harsh environment for a certain period of time and sometimes only one hair might be collected and used for further analysis. These interfering factors would have a significant impact on the estimation of age for a real forensic sample. Accordingly,

this assay only provides an estimate range rather than an exact value of the time since the estimation may be affected by the factors listed above. More attention needs to be paid when applying this equation on forensic type samples and further investigation with a larger amount of subjects, a longer time frame is required to confirm the findings. Further research should be carried out with a focus on the effects of various environmental factors and the influence of the chemicals in hair dye or perm on the aging of hair samples.

Chapter 6 General discussion and future work

It is likely that body fluid identification and other applications on forensic science using RNAs will undergo a revolution in the near future. The technology for molecular biology is becoming increasingly sensitive and robust, which might allow forensic scientists to discover more crucial information from biological evidence. The methods of applying analysis of RNAs on forensic identification have been addressed in many studies over the last few decades. Some of them have revealed the forensic potential of RNA for identification of body fluids and evaluation of the time since deposition of a biological material from the crime scene [7, 177, 192, 404]. The research, presented here, explored several possible methods and uses of RNAs in forensic science. Initial attempts were made to establish analysis models not only for identifying blood and saliva using real-time RT-LAMP technology, but also determining the time since a hair was plucked by the relative quantification of RNA.

6.1 Overall summary

6.1.1 Identification of blood and saliva by LAMP

Identification of body fluids in a forensic investigation can provide crucial information. The identification can be aided by LAMP technology, basing on its high sensitivity and specificity. In this study, LAMP was evaluated and confirmed as a novel method for body fluid identification. A model was established for blood and saliva which were the most common body fluids found at crime scenes. One marker (*HBB*) was developed for the identification of blood by real-time RT-LAMP and two other markers (*HTN3* and *KRT4*) were developed for saliva, while *18S* rRNA was used as the

internal control. Both *HBB* and *HTN3* markers showed high specificity in blood and saliva respectively. This means both markers are capable of use for identification of blood/saliva based on our study. Another marker, *KRT4* was found not only in saliva samples but also in menstrual blood, sweat, and vaginal secretion samples, indicating that *KRT4* marker might not be accurate for saliva identification. Yet, it is still of use when the alleged fluid is saliva, blood, or sweat. *KRT4* could also be used for confirming saliva samples along with *HTN3*.

This study showed that blood and saliva can be successfully identified and, so for, without producing false positives. Nonetheless, this conclusion should be treated with reservation when applied in forensic analysis due to the limited sample size and body fluid types used in the study. Various kind of body fluids or their mixtures may be found at the crime scene, which requires additional extensive validation studies. More samples for each body fluid and more types of body fluids should be further tested to confirm the specificity of these markers. The specific mRNA may be also expressed in cancer cells from abnormal gene expression or even in some healthy tissues from normal gene regulation. Illegitimate transcription of mRNA could happen and lead to false positive. In fact, even it can be proved that the selected marker is 100% sensitive for a certain body fluid, the possibility that the specific target mRNA might illegitimately transcribed from a non-specific tissue cannot be eliminated. Consequently, a false positive result might be attributed to illegitimate transcription. To avoid such false results, instead of using only one marker, more specific markers should be used to identify a certain body fluid to reduce the effects of non-specific expression of a single marker. Roeder *et al.* suggested that using a minimum of five mRNA markers per body fluid is required and also demonstrated a scoring method for

body fluid identification [146]. We agree with this opinion as it not only helps to reduce the false positive rate but also increases the statistical power.

Sensitivity was also evaluated for both *HBB* and *HTN3* markers. First of all, it is worth noting that there was an extraordinary disparity in the limit of detection between the two markers. Based on our study, it was deduced that the limit of detection for blood with *HBB* marker reached 2×10^{-6}µl for liquid blood, but only 1µl for saliva identification with *HTN3* marker. The difference of sensitivity between the two markers might be attributed to several reasons. First, the levels of gene expression of the two markers are different, which directly impacts the sensitivity. Second, the efficiency of LAMP reaction also has significant influence on the result. Besides, one millilitre of blood contains 4.3×10^{6} to 5.2×10^{6} erythrocytes [405] which is the source of *HBB* mRNA. On the other hand, one millilitre of saliva contains less than 200 columnar epithelial cells [406]. These cells are found in salivary glands and are considered as the source of *HTN3* mRNA in saliva. As a consequence, identifying blood with *HBB* marker using LAMP can be much more sensitive than identifying saliva with *HTN3* marker.

Furthermore, the assay was compared with the methods routinely used for body fluid idnetification in forensic practice. In this study, we focused on blood and saliva. Several studies have been made to validate the sensitivity of these methods. Although the results are slightly inconsistent, it is demonstrated that the sensitivity of identification of blood using LAMP with *HBB* marker is at the same level comparing with the KM blood test – a method that is considered as the most sensitive presumptive blood test and is frequently used in forensic investigations. It is also reported that identification of saliva using LAMP with *HTN3* marker roughly reaches

the same level of sensitivity of the Phadebas® Quantitative test and RSID™-saliva test (both have been widely applied for forensic practice).

Although our assay may not be good enough to replace these methods currently used, the result still indicates that LAMP reaction is capable of body fluid identification. With the optimization of primers and LAMP reaction, the sensitivity and specificity of this assay can be further boosted. Thus, we believe that this assay has great potential for body fluid identification in the future.

LAMP reaction may not be the most convenient method for body fluid identification comparing with other chemical presumptive tests, such as KM test for blood identification, but there are many advantages making LAMP a novel and practial method for body fluid identification, including:

1. LAMP reaction can be executed in one single temperature, which makes it possible to be carried out by a simple portable heating block for body fluid analysis at crime scenes. Comparing with traditional PCR, this method is not necessary to denature DNA at the initial stage. It also reduces the required time to complete the reaction since there is no need to provide heating-cooling control. The whole analysis for the source of body fluid by LAMP can be accomplished in 15-60 minutes.

2. It has high specificity for body fluid identification by LAMP. At least four primers based on six distinct regions of the target sequence are required for LAMP. In contrast to PCR which uses only two primers, LAMP is much more specific.

3. It is time and labour-saving by applying RT-LAMP in the analysis of body fluid. For RNA analysis, it is usually required to reverse transcribe RNA into cDNA in advance before subsequent amplification, which usually takes at least 30 minutes and may in turn can incur more cost and labour. In our method, reverse transcription and

LAMP reaction are performed simultaneously in the same tube (RT-LAMP). The cooperative multitasking reaction not only shortens the required time for the procedure of body fluid identification but also eliminates the possibility of contamination because RNA was used directly for LAMP without opening the lid. Another benefit is that there is little or no loss of nucleic acid, which usually occurs in the reverse transcription procedure. RNA can be used more efficiently in the analysis of body fluid by RT-LAMP.

4. The yield of LAMP reaction is considerably higher than PCR amplification due to the specific nature of LAMP reaction (for example, denaturing the double-stranded DNA before annealing and thermal cycling are not required) [407]. Its corresponding by-product material, pyrophosphate, is released and allows easy visualization by the naked eye thanks to its visible turbidity. LAMP product can be also estimated by the naked eye using simple fluorescent dye. This makes LAMP more convenient to be applied at crime scenes. This study has also found that LAMP reaction can be monitored in real-time either by estimating the turbidity or the fluorescent signals produced by the reaction from the by-product material and fluorescent dye (real-time RT LAMP). In this way, the source of body fluid can be identified once the signal reaches the threshold.

Although there are several advantages of applying LAMP to body-fluid identification as mentioned above, there are also some intrinsic drawbacks. First, the inability to multiplex targets makes it less efficient in detecting multiple body fluids simultaneously. Second, in some cases (such as further analysis using electrophoresis), the requirement of opening reaction tubes post-amplification would increase the possibility of contamination, which can be disastrous in forensic investigations. In terms of the primer design, a specific software is required. As there are at least 4

primers used for detecting 6 distinct regions, the restriction on the primer design increases. Finally, the LAMP products are large fragments in various lengths and structures, which are difficult to utilise in other molecular biology applications (such as sequencing). In spite of these disadvantages, LAMP is still useful to body fluid identification owing to the advantages listed above.

To summarize, a systematic, cost-effective, rapid and labour-saving confirmatory assay was developed. It was applied successfully to the putative identification of blood from 21 non-probative forensic samples. This is the first application of LAMP for body fluid detection in a forensic science context. With the advantages above, the source of body fluid can be identified with high specificity, sensitivity and rapid amplification by real-time RT LAMP.

6.1.2 Dating a plucked hair

Determining the time when the biological evidence was deposited at the crime scene is useful for establishing the timetable of the crime in forensic investigation. The possibility of estimating the time since deposition of a plucked hair was investigated by examining the relative degradation between two different RNA species (*18S* rRNA and *ß-actin* mRNA), using real-time PCR quantification. A plucked hair was chosen as the target, it may not be the most common type of hair evidence (shed hair being more common) but it is often found after violent altercations. Therefore it can be indicative of activity and serve as source level evidence. Since it contains intact genetic material from the hair root cells there is an adequate amount of nucleic acid for analysis. A linear polynomial (TSP = 0.069 × dCq + 14.445, r^2=0.78) from linear regression and a second-order polynomial (TSP = -0.002 × dCq^2 + 0.193 × dCq + 13.436,

r^2=0.98) from quadratic regression respectively were successfully established as a model for age estimation of the plucked hair. The quadratic regression is better than the linear regression in describing the correlation between the average dCq value and the time since the hair was plucked. It is also found that the relationship between the average dCq value and the time could be sorted by another linear equation (TSP = 0.168 × dCq + 13.406, r^2=0.99) within 21 days. After 21 days, the time-wise trend of the change of the average dCq value became less significant and gave unreliable estimates. Hampson *et al.* proposed another polynomial for the age of the hair by monitoring the relative expression ratio of the two RNA species (*18S* rRNA and *ß-actin* mRNA) and showed that the evaluation could only be made within 60 days [192]. The limitations of time range in both assays varied widely (21 days v 60 days). It was believed that the difference might be caused by the unreliable screening method (microscopy) from hair collection and the amount of hair used in one tube (3 hairs v 10 hairs) for each test (tube). The unreliable selection at the beginning of the experiment and limited material tested might increase the variety of the result. Comparing both studies, Hampson *et al.* gave a conclusion which is similar to this study in that the assay might have time limitation for the estimation of the age and could be only used to estimate a range, rather than an exact value of the time.

The sample size used in this study was small, so investigations on more samples are required before further application. Besides, although polynomial equations were proposed in this study to evaluate the age of the plucked hair, several factors (such as chemicals used for hair dye or perm, various uncontrolled environmental conditions, and the uncertainty of the actual phase of the plucked hairs) might affect the degradation progress, introduce more uncertainty for the test, and accordingly increase the standard deviation of the dCq value for each time point.

There is a practical value of dating the time since the hair was plucked. In spite of the limitations and many impact factors in this assay, the proposed polynomials could still provide corroborating evidence in certain scenarios. It is worth noting that it might be arbitrary to evaluate the age of evidence based only on this assay. It is suggested to join several methods collaboratively involving biology, anthropology, or archaeology on determination of the age of biological materials in different time frames.

6.2 Further future work

The application of real-time reverse transcription loop-mediated isothermal amplification to identify the presence of a specific body fluid as a proof-of-concept model was developed in this study, using blood and saliva. The result showed the potential to apply this method to identification of other body fluids including common body fluids, such as urine and sweat, as well as rare body fluids, such as nasal mucus. It is worth further research to extend the application to more body fluids. In order to achieve better specificity, at least five specific markers for each body fluid are required. More research on the selection of markers for further application should be done.

DNA-STR analysis is usually carried out after presumptive test or the confirmation of the body fluid and it may require more time to extract DNA afterwards. Besides, forensic evidence may be consumed for the following DNA extraction. Several studies have been proposed on total DNA/RNA co-extraction to use in the same forensic samples [408, 409]. This could work with our assay cooperatively, which will not only reduce the time and labour for further body fluid identification and STR analysis, but also save precious samples for the analysis of reconfirmation.

The methods in this project could further be applied to different tissues, which can be the targets in some cases. For example, in car accidents, tissues can be important evidence to connect the death of the victim with the suspect, if the tissue on the suspect's tire is confirmed to come from the victim by STR. However, the suspect may argue that the STR profile is from victim's blood and the car accident is not the fatal reason causing the death of the victim. If the sample is proven as a brain tissue, it could help reconstruct the crime scene in such scenario. These methods can also potentially be applied to future identification of other tissues. Moreover, siRNA, which have been proved to be tissue specific, may be used to identify different body fluids besides mRNA.

Some modified methods for detection of LAMP products were developed in different ways. For example, Watthanapanpituck *et al.* demonstrated a method to identify human DNA in forensic evidence by LAMP combined with a colourimetric gold nanoparticle hybridization probe [246]. Mohon *et al*. introduced that pre-addition of hydroxynapthol blue (HNB) in the LAMP reaction caused a distinct colour change. Xie *et al*. developed an electrochemical method for LAMP [410] and another method to monitor the LAMP by pH meter [411]. Some problems encountered in the application of LAMP are also evaluated, such as nonspecific amplification from randomly produced nicks. Mitsunaga *et al*. applied an improved LAMP using RecA and a restriction enzyme for enhanced amplification specificity [221]. Wang *et al.* also reported that the addition of dimethyl sulfoxide (DMSO) could improve specificity and sensitivity in LAMP reaction [284]. These improved methods for LAMP can also be tested in body fluid identification in the future.

Singleplex LAMP can only check one marker in one reaction, so at least two tubes (for internal control and specific marker respectively) are required for a

singleplex test. Multiplex real-time RT LAMP for different markers of the same or different body fluids can also be a direction of research in the future. For example, if blood markers and menstrual blood markers can be detected at the same time in one tube by a multiplex LAMP reaction, a fluid in question collected from the sexual case can be confirmed for its nature. However, there is difficulty for multiplex amplification due to the polymorphic length of LAMP products. In recent years, some methods for improvements have been tested and some resolutions have been discovered. The simplest method is cutting the amplified fragments by specific restriction enzyme [412]. Aonuma *et al*. observed the different LAMP products by directly labelled different fluorescence [413]. Xu *et al*. developed a LAMP chip for multiplex LAMP [273] and Tanner *et al*. developed a method named "Detection of Amplification by Release of Quenching" (DARQ) [414]. Khamlor *et al.* demonstrated a multiplex LAMP assay using polyethylenimine for precipitation of LAMP products [415]. Based on these previous studies, body fluid identification by multiplex LAMP can be an area worth experimenting in the future.

Although LAMP reaction can be carried out with a simple heater, it requires chemical reagents preserved at low temperature. It also needs to be conducted with qualified facilities in experimental environment. In additional, extracting RNA before the RT-LAMP reation is time-consuming. As a result, RNA extraction does pose a practical problem – it can hardly be performed at crime scenes, which makes it almost impossible to carry out the identification of body fluids by LAMP. To overcome this, instead of extracting RNA, the sample can be treated by a simple heating method [416] and then be used directly for the following LAMP reaction (direct LAMP). Nie *et al.* found that the direct RT-LAMP reaction can potentially be developed for the screening of EV71 infection [417]. Direct LAMP could not only turn this experimental method

into reality, but also reduce the time for the whole reaction. It saves the step of RNA extraction which is inconvenient and time-consuming. It can be a great step forward in forensic body fluid identification if all works well.

Regarding the age estimation of biological evidence, the assay used for the plucked hair has also been applied on dating the bloodstains and has proven its potential for the age estimation. A systematic method was developed accordingly, which might also be applicable to dating the age of other human tissues in forensic science. For example, this could be used for the estimation of time since death by estimating the expression of specific mRNA in human brain [195]. Sampaio-Silva *et al.* reported a method for estimation of the post mortem interval by profiling of RNA degradation [418].

The differential rate of degradation between two kinds of RNAs was utilised to estimate the age of the pluck hairs in this study. As mentioned, environmental conditions have a significant influence on the degradation progress and subsequently impact the result of this study. Chemicals left on hairs may also affect the degradation of RNA. Due to these factors, this assay can only provide an approximation of the accurate time since it is impossible to avoid these influences. Future research should focus on the effects of various environmental factors. Investigation of the sample donors on the use of chemical for hair care or styling should also be made and recorded to monitor their influence on RNA degradation. A larger sample size should be collected and used to reduce the deviation of the result. The uncertainties during the actual growth phase of the hairs used for this test also increase the deviations. Therefore, more hair samples (using more hairs, instead of three hairs) with follicles for RNA extraction in one tube for one test are suggested to reduce the effect.

Age estimation of biological samples is based on the idea that mRNA decays faster than rRNA. In this study, *ß-actin* mRNA was chosen as a target because it is expressed at relatively constant levels in all cells. If *ß-actin* mRNA can be replaced with another body fluid-specific mRNA successfully, the two main goals of the thesis, identification and age estimation of body fluids with RNA markers, may be achieved simultaneously. For example, *HBB* mRNA was used as a blood-specific marker for the identification of blood and it showed highly sensitivity according to this study (see Section 3.3.3). If 18S rRNA and *HBB* mRNA could be analysed at the same time from an alleged stain, it could be confirmed that it is a bloodstain and the time of its deposition can also be evaluated.

Beside 18S rRNA, only a single calibrator (*ß-actin* mRNA) was used for age determination in this test. Adding more aging markers (slow-degrading mRNA) could improve age estimation significantly with a higher degree of confidence. Instead of adding other housekeeping genes, body fluid-specific markers could be added to increase the statistical power for age estimation and to confirm the type of body fluid. The same method might be applicable to saliva by replacing *HBB* with *HTN3* mRNA and more different body fluids could also be analysed in the same way.

As real-time RT-LAMP was used for the RNA quantification [419], it might also be applicable to quantification of these RNA markers used for age determination. Theoretically, the rate of DNA amplification using real-time RT-LAMP is faster than using real-time quantitative PCR, since repetitive heating and cooling is not required for LAMP reaction. LAMP could therefore be a novel method for age determination as well.

The current project aimed to gain more knowledge and to provide an improved way of RNA application in forensic science. Although it is still necessary to further evaluate the validity of the proposed methods, this work has provided insight into the possible applications for the crime scene investigation and has paved the way for further research in forensic science.

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Appendix 1

Ethics

The research was carried out following an application to the KCL School of Health and Biomedical sciences Research Ethics Committee. In the following pages are included the Ethics application form, the information sheet for volunteers and the consent form.

NHS
National Research Ethics Service
South East London REC 3
(formerly King's College Hospital Research Ethics Committee)
1st Floor Camberwell Building
King's College Hospital
94 Denmark Hill
London SE5 9RS

Telephone: 020 3299 3923
Facsimile: 020 3299 5085

06 September 2010

Mr Chih-Wen Su
PhD student
Flat 5
102 Long Lane
London SE1 4BN

Dear Mr Su

Study Title: **The use of RNA markers in Forensic Science**
REC reference number: **10/H0808/94**

Thank you for your letter of 18 August 2010, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where the only involvement of the NHS organisation is as a Participant Identification Centre (PIC), management permission for research is not required but the R&D office should be notified of the study and agree to the organisation's involvement. Guidance on procedures

for PICs is available in IRAS. Further advice should be sought from the R&D office where necessary.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Investigator CV		18 June 2010
Protocol	1.0	15 June 2010
Academic Supervisor CV - Barbara Daniel		18 June 2010
REC application		15 June 2010
Covering Letter		18 August 2010
Summary/Synopsis	1.0	15 June 2010
Advertisement	1.0	15 June 2010
Participant Information Sheet	2.0	19 August 2010
Participant Consent Form	2.0	19 August 2010
Evidence of insurance or indemnity		01 August 2010
Referees or other scientific critique report		15 June 2010

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

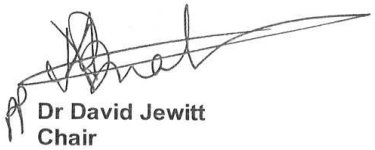
The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email

referencegroup@nres.npsa.nhs.uk.

10/H0808/94	Please quote this number on all correspondence

Yours sincerely



Dr David Jewitt
Chair

Email: juliet.kirk-buaku@nhs.net

Enclosures: "After ethical review – guidance for researchers" *SL- AR2 for other studies*

Copy to: *Dr. Barbara Daniel, King's College London*

INFORMATION SHEET FOR PARTICIPANTS

REC Name: South East London REC 3 REC Number: 10/H0808/94

The use of RNA markers in Forensic Science – Version 1.0 (15/6/2010)

We would like to invite you to participate in this postgraduate research project. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

I am a PhD student in Department of Forensic Science & Drug Monitoring, King's College London. As part of my PhD project, I am carrying out research on identification of body fluids by using RNA markers.

- Aims of the research and possible benefits
  The research involves an investigation to evaluate the use of RNA markers to identify different body fluids, including blood, semen, saliva and sweat. These RNA markers can be used as a useful tool in forensics which is confirmatory, sensitive and specific for individual body fluid. Moreover, some researches indicate that it is possible to estimate the time when the body fluids were deposited. The different RNA markers will be also used to evaluate this possibility.

- Who you are recruiting (including exclusion criteria)
  English-speaking volunteers will be recruited in order to ensure a full understanding of the study and the procedures employed. Healthy volunteers above 18 age, both male and female will be included. Pregnant women will be excluded from the study as mRNA levels can be affected.

- What will happen if the participant agrees to take part (when, where, how long etc)
  In order to take part in the study we will ask you to sign a consent form which will be provided. You do not need to complete any questionnaire since your personal information and medical history is not required in this study.
  After you decide to participate in the study, your body fluid sample (blood, semen,

saliva or sweat) will be taken. 10ml whole blood sample will be collected using phlebotomy procedure which will be performed by qualified personnel. The procedure will take place in a clean, private first-aid room in the department. The semen sample will be collected using a sterile plastic container. For the saliva samples you will be asked to rinse your mouth before and then 2ml saliva will be collected in a sterile plastic container. For sweat samples you will be ask to exercise around 20 minutes and then collect 1ml of your sweat in a sterile container. All the equipment used in the procedure are sterile and disposable to minimize the risk of infection.

- Any risks (e.g. need for disclosure of information to a third party, possibility for distress, potential adverse reactions)
  The possible risk of infection from the procedure is minimal. You may experience pain during blood collecting procedure but it usually does not cause overt pain. If you feel uncomfortable with phlebotomy procedure you can request that the procedure shall stop and withdraw yourself from your participation from the study.

- Possible benefits (it is good practice to offer participants a copy of the final report)
  Your participation in this study might be of invaluable help to the progress in medico-legal or criminal investigation, but will not benefit you directly. You will receive a copy of the final research project if you are interested.

- Arrangements for ensuring anonymity and confidentiality. To ensure compliance with the Data Protection Act 1998 participants must be informed of what information will be held about them and who will have access to it (this relates to information that is identifiable or could potentially be linked back to an individual).
  To avoid concerns over your privacy, the source of each donation will be kept anonymous and confidential. Each sample is given an individual code number that will only be known to the investigators and recorded in a secure computer, thus not be traceable to the original donor.
  The data will be published at the end of the research in a thesis and scientific publication, with the original donors kept anonymous. Individual's profiles will not be included in this report. Participants will be allowed to look through the report once it is complete.

- Name and contact details of the researcher (please note that the use of personal contact details can cause problems).

  Please contact Chih-Wen Su or Dr. Barbara Daniel on the details below if you have any questions.

  Chih-Wen Su
  Department of Forensic Science and Drug Monitoring
  Phone: 0207 8484489
  Email: chih-wen.su@kcl.ac.uk
  Address: Department of Forensic Science and Drug Monitoring
  4th floor, Franklin-Wilkin Building
  150 Stamford Street
  London SE1 9NH

  Dr. Barbara Daniel
  Department of Forensic Science and Drug Monitoring
  Phone: 0207 8483841
  Email: barbara.daniel@kcl.ac.uk
  Address: Department of Forensic Science and Drug Monitoring
  4th floor, Franklin-Wilkin Building
  150 Stamford Street
  London SE1 9NH

It is up to you to decide whether to take part or not. If you decide to take part you are still free to withdraw at any time and without giving a reason.

If this study has harmed you in any way you can contact King's College London using the details below for further advice and information:

Su Chih-Wen chih-wen.su@kcl.ac.uk
Dept. of Forensic Science and Drug Monitoring 0207 8484489

CONSENT FORM FOR PARTICIPANTS IN RESEARCH STUDIES

Version 1.0 (15/6/2010)

Please complete this form after you have read the Information Sheet and/or listened to an explanation about the research.

Title of Study: The use of RNA markers in Forensic Science

KING'S College LONDON
University of London

- **Thank you for considering taking part in this research. The person organizing the research must explain the project to you before you agree to take part.**

- **If you have any questions arising from the Information Sheet or explanation already given to you, please ask the researcher before you decide whether to join in. You will be given a copy of this Consent Form to keep and refer to at any time.**

- ***I confirm that I have read and understood the information sheet for the above study.***

- ***I confirm I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.***

- ***I understand that my participation is voluntary and I am free to withdraw at any time, without giving any reason.***

- ***I understand that if I lose the capacity to consent during study after having given informed consent, I will be withdrawn from the study and my data retained and further use of identifiable data/tissue already available will be used for the research purpose of this study. No further data or tissue would be collected or any other research procedures carried out on or in relation to me.***

- ***I consent to donate (please tick at least one of the followings):***
  - ☐ ***10 mL whole blood***
  - ☐ ***2 mL semen***
  - ☐ ***2mL saliva***
  - ☐ ***1mL sweat***

- **I understand that if I decide at any other time during the research that I no longer wish to participate in this project, I can notify the researchers involved and be withdrawn from it immediately until the samples have been analysed.**

- ***I consent to the processing of my personal information for the purposes explained to me. I understand that such information will be handled in accordance with the terms of the Data Protection Act 1998.***

Participant's Statement:

I _____

agree that the research project named above has been explained to me to my satisfaction and I agree to take part in the study. I have read both the notes written above and the Information Sheet about the project, and understand what the research study involves.

Signed Date

Investigator's Statement:

I _____

confirm that I have carefully explained the nature, demands and any foreseeable risks (where applicable) of the proposed research to the volunteer.

Signed Date

Appendix 2

RNA concentration for each sample using Nanodrop®.

Sample ID	1st measurement (ng/µl)	2nd measurement (ng/µl)	3rd measurement (ng/µl)	Average (ng/µl)
Blood 1	4.6	4.5	4.9	4.7
Blood 2	4.8	5.2	5.0	5.0
Blood 3	5.8	6.8	7.0	6.5
Semen 1	35.5	35.5	35.7	35.6
Semen 2	52.8	52.3	52.7	52.6
Semen 3	54.0	53.9	54.4	54.1
Saliva 1	3.4	3.5	3.4	3.4
Saliva 2	7.4	7.7	8.1	7.7
Saliva 3	4.5	4.8	5.0	4.8
Menstrual blood 1	14.5	20.1	16.6	17.1
Menstrual blood 2	17.6	17.9	18.3	17.9
Menstrual blood 3	11.6	8.5	9.4	9.8
Sweat 1*	6.3	5.3	12.5	8.0
Sweat 2*	4.5	5.4	3.7	4.5
Sweat 3*	5.5	11.5	7.6	8.2
Urine 1*	8.7	8.7	9.8	9.1
Urine 2*	7.6	4.2	4.9	5.6
Urine 3*	12.7	13.0	7.6	11.1
Vaginal secretions 1	11.1	11.9	10.4	11.1
Vaginal secretions 2	21.1	20.8	18.2	20.0
Vaginal secretions 3	13.4	15.6	12.9	14.0

* RNA was extracted from samples concentrated from 10 ml to 1 ml before the extraction to obtain more starting materials.

Appendix 3

Tt (min) of real-time RT-LAMP (18S) with different body fluids performed in triplicate using one sample

ID	Tt(min)							
	Blood	Saliva	Semen	Menstrual blood	Sweat	Urine	Vaginal secretions	NC
1	42.1	35.3	41.7	40.2	44.2	42.0	43.0	-
2	41.8	37.0	42.6	40.6	41.7	43.5	43.8	-
3	38.2	35.6	38.7	36.5	42.4	39.3	42.1	-
Average ±SD[a]	40.7 ±2.2	36.0 ±0.9	41.0 ±2.0	39.1 ±2.3	42.8 ±1.3	41.6 ±2.1	43.0 ±0.9	

[a] SD represents the standard deviation.

Tt (min) of real-time RT-LAMP (HBB) with different body fluids performed in triplicate using one sample

ID	Tt(min)							
	Blood	Saliva	Semen	Menstrual blood	Sweat	Urine	Vaginal secretions	NC
1	38.5	-	-	44.2	-	-	-	-
2	31.3	-	-	37.8	-	-	-	-
3	29.4	-	-	39.7	-	-	-	-
Average ±SD[a]	33.1 ±4.8	-	-	40.6 ±3.3	-	-	-	

[a] SD represents the standard deviation.

Tt (min) of real-time RT-LAMP (18S) with 7 body fluids from 6 different individuals

sequence	Blood	Saliva	Semen	Menstrual blood	Sweat	Urine	Vaginal secretions
1	42.5	45.2	41.6	35.4	55.6	46.5	44.0
2	45.4	39.0	42.8	34.8	54.2	41.5	45.4
3	37.7	48.2	39.4	44.3	50.0	39.5	43.0
4	49.9	38.4	37.7	39.8	41.9	45.3	47.5
5	46.8	43.2	47.2	43.9	57.2	44.1	46.0
6	45.9	38.8	42.3	46.9	47.9	52.6	44.4
Average ±SD[a]	44.7 ±4.2	42.1 ±4.1	41.8 ±3.3	40.9 ±5.0	51.1 ±5.7	44.9 ±4.5	45.1 ±1.6

[a] SD represents the standard deviation.

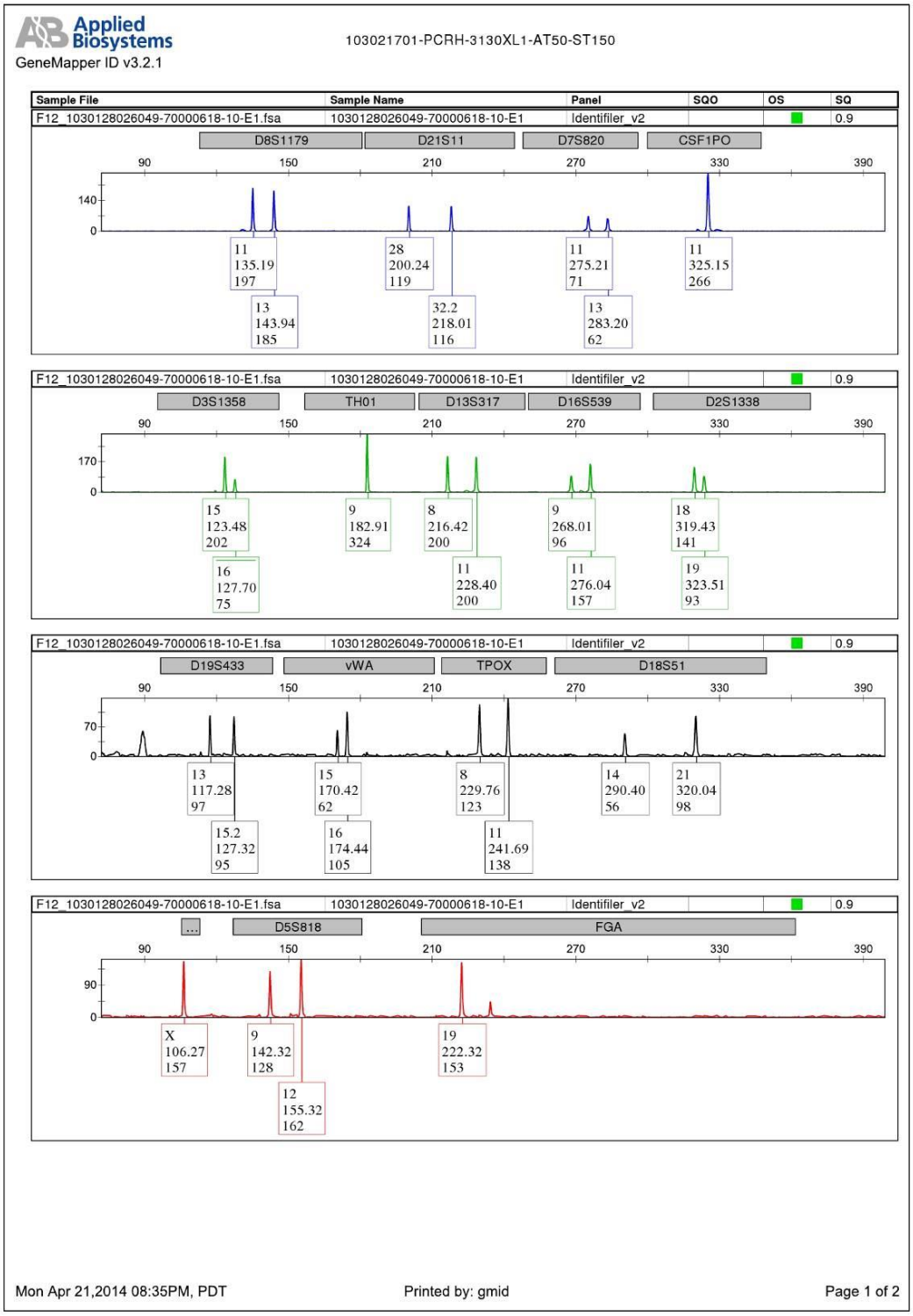
Tt (min) of real-time RT-LAMP (HBB) with 7 body fluids from 6 different individuals

Sequence	Blood	Saliva	Semen	Menstrual blood	Sweat	Urine	Vaginal secretions
1	32.4	-	-	55.4	-	53.3[a]	-
2	40.6	-	-	49.8	-	50.6 [a]	-
3	35.4	-	-	44.7	-	-	-
4	31.9	-	-	38.3	-	-	-
5	39.7	-	-	48.5	-	-	-
6	41.0	-	-	43.0	-	-	-
Average ± SD[c]	36.8 ± 4.1	-	-	46.6 ± 5.9	-	NA[b]	-

[a] Urine samples were collected from the females during the menstrual period.
[b] The symbol "-" represents no LAMP product was detected and "NA" represents that the result was not analysed.
[c] SD represents the standard deviation.

Appendix 4

Partial profiles of the non-probative forensic samples (Sample 3).



Applied Biosystems
GeneMapper ID v3.2.1

103021701-PCRH-3130XL1-AT50-ST150

Sample File	Sample Name	Panel	SQO	OS	SQ
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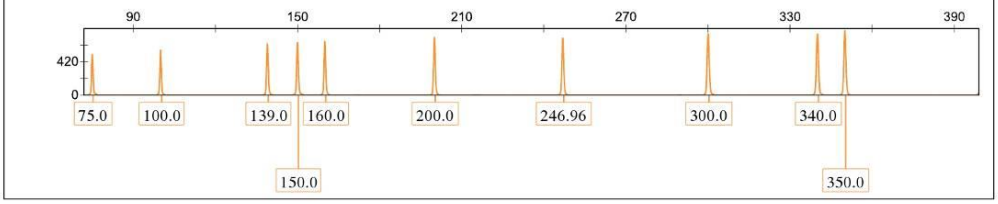
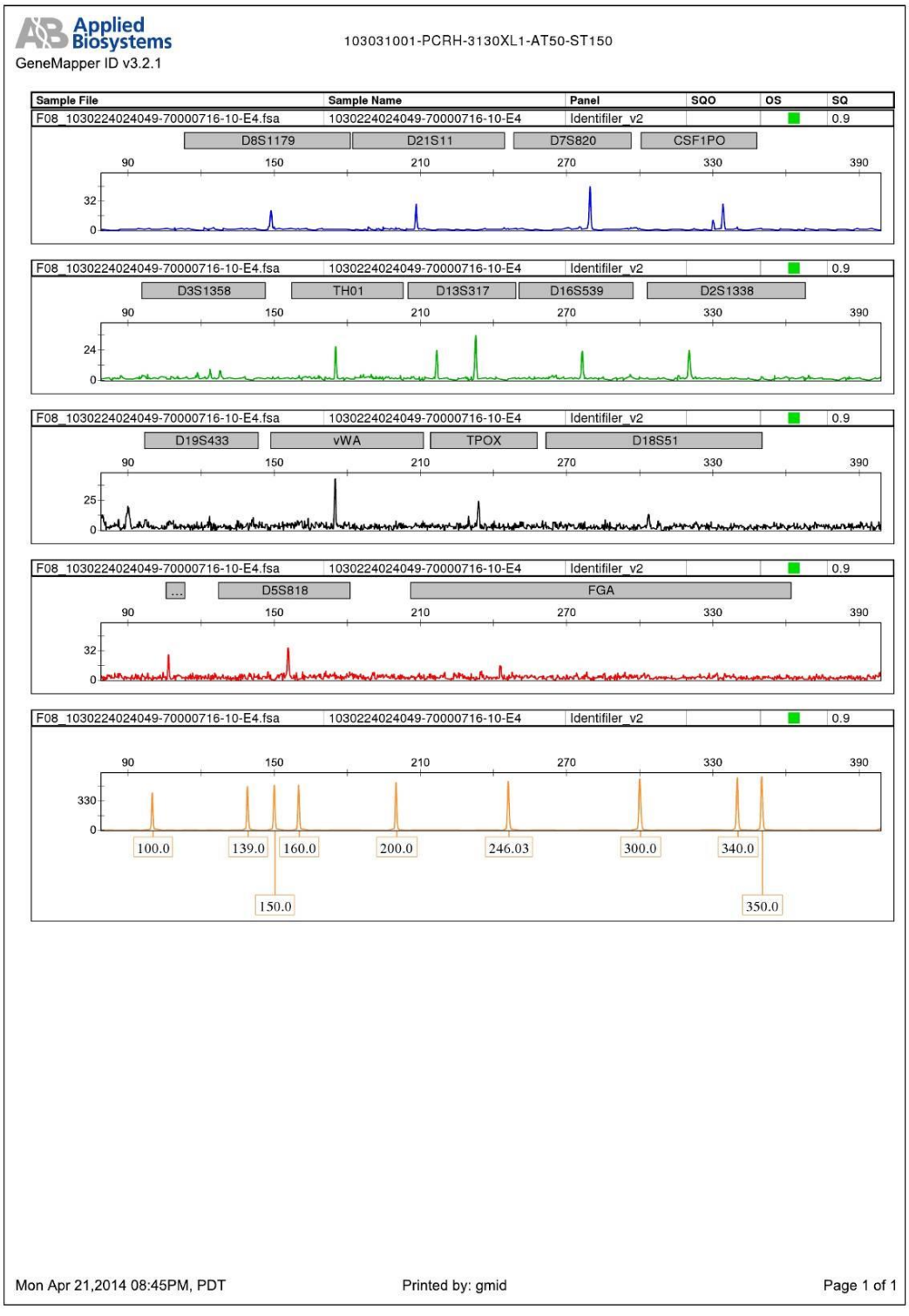
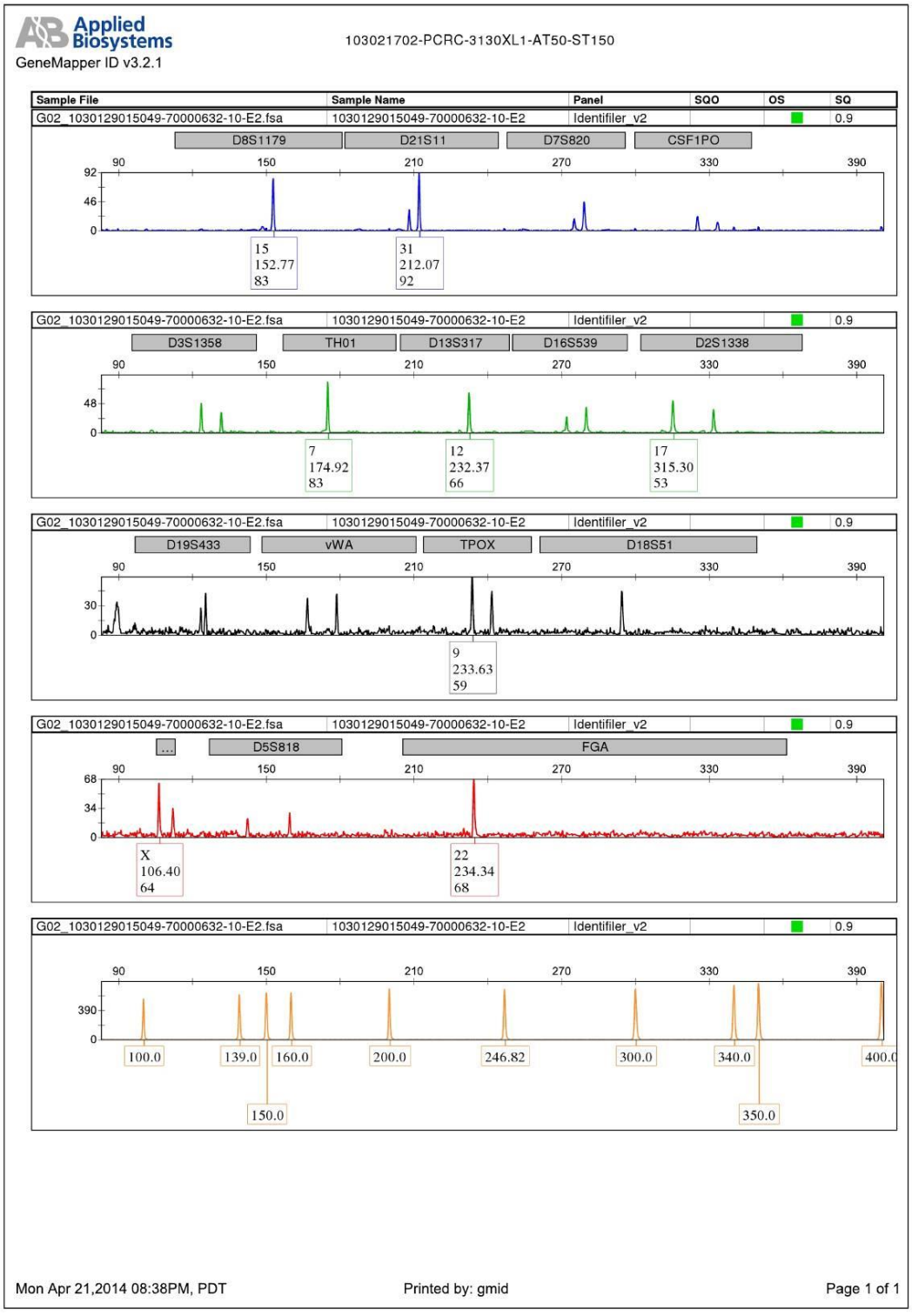


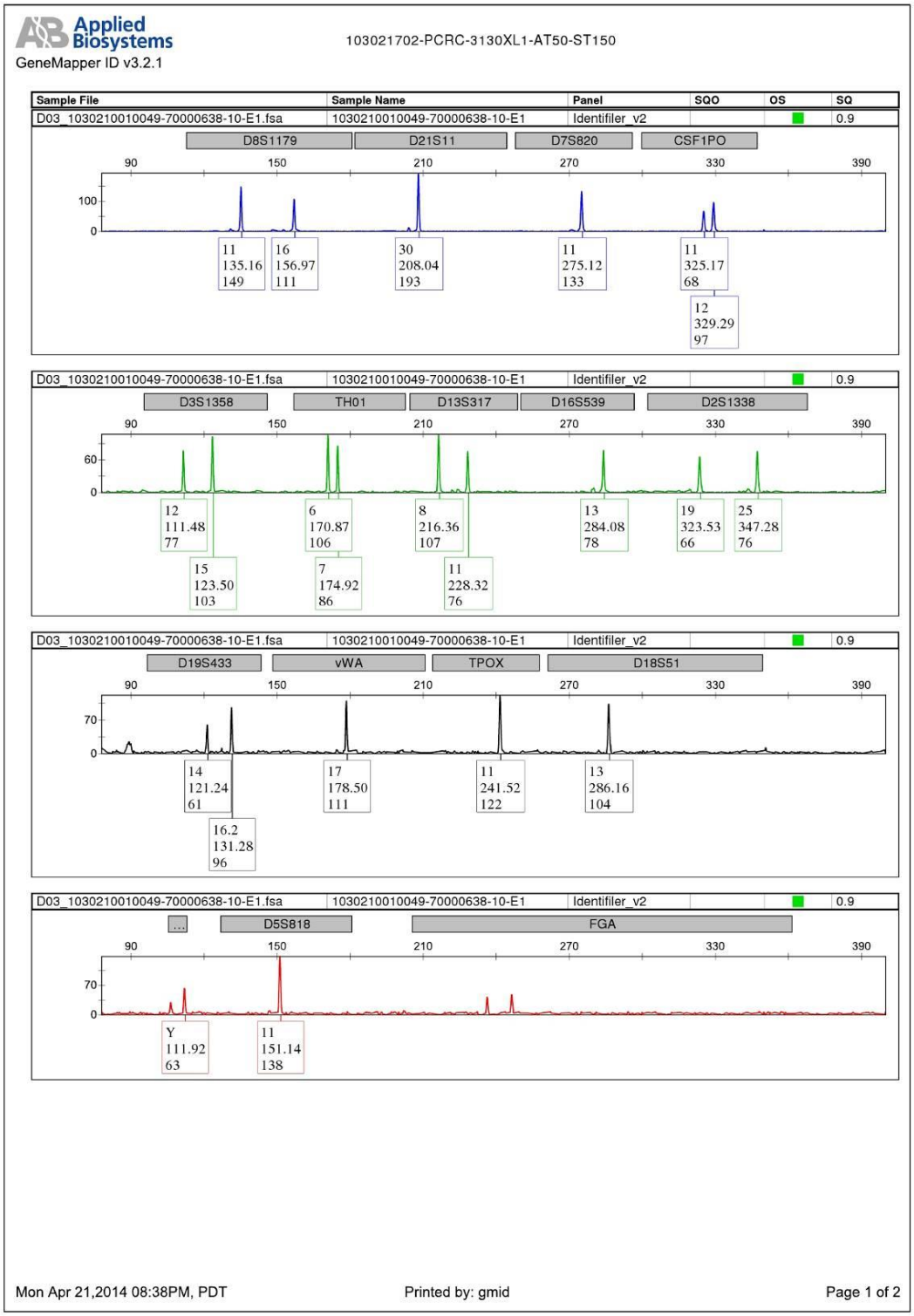
Partial profiles of the non-probative forensic samples (Sample 5).



Partial profiles of the non-probative forensic samples (Sample 8).



Partial profiles of the non-probative forensic samples (Sample 9).



Applied Biosystems
GeneMapper ID v3.2.1

103021702-PCRC-3130XL1-AT50-ST150

Sample File	Sample Name	Panel	SQO	OS	SQ
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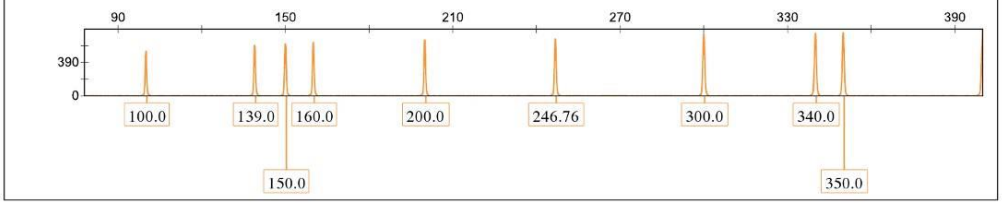
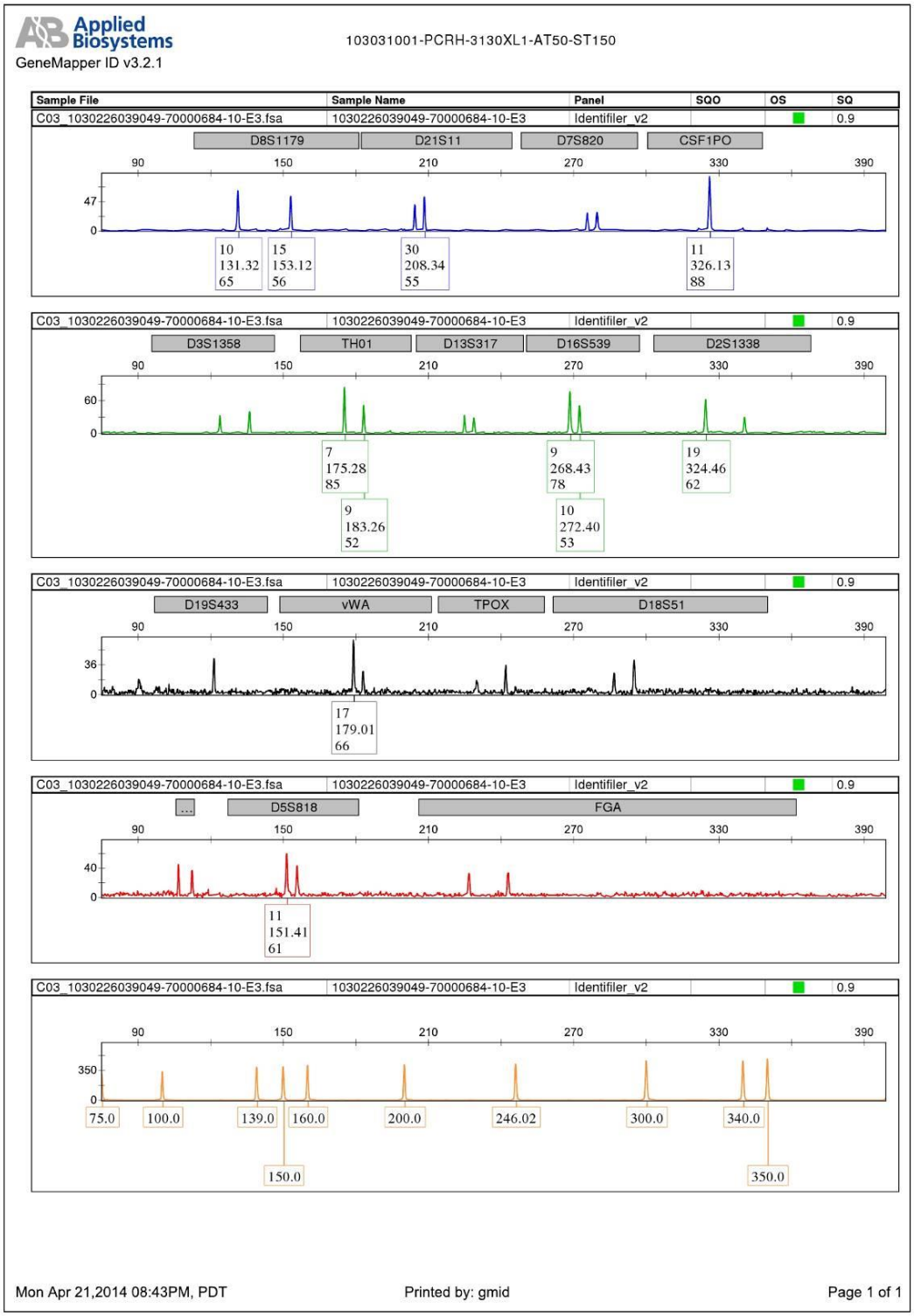
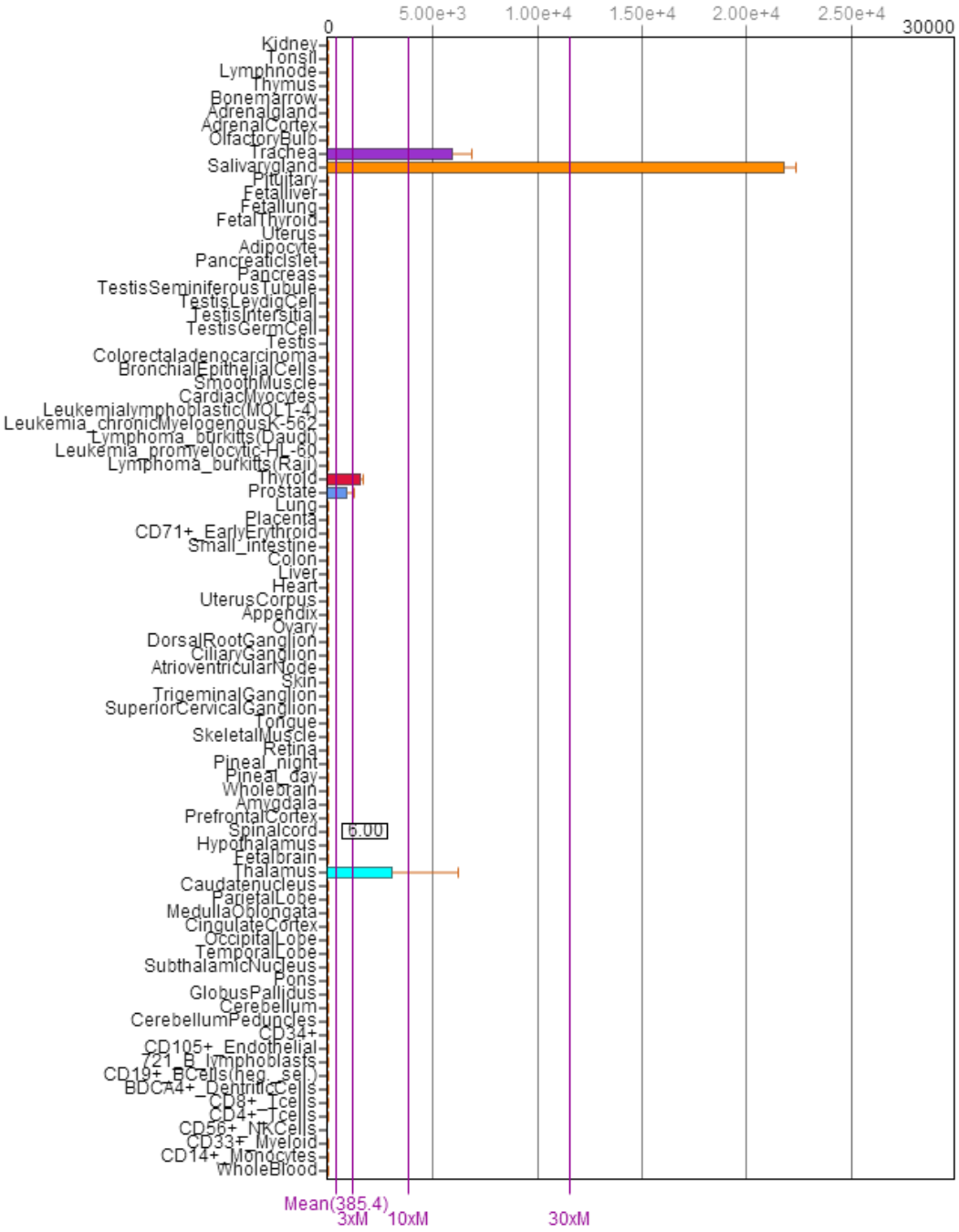


Partial profiles of the non-probative forensic samples (Sample 10).

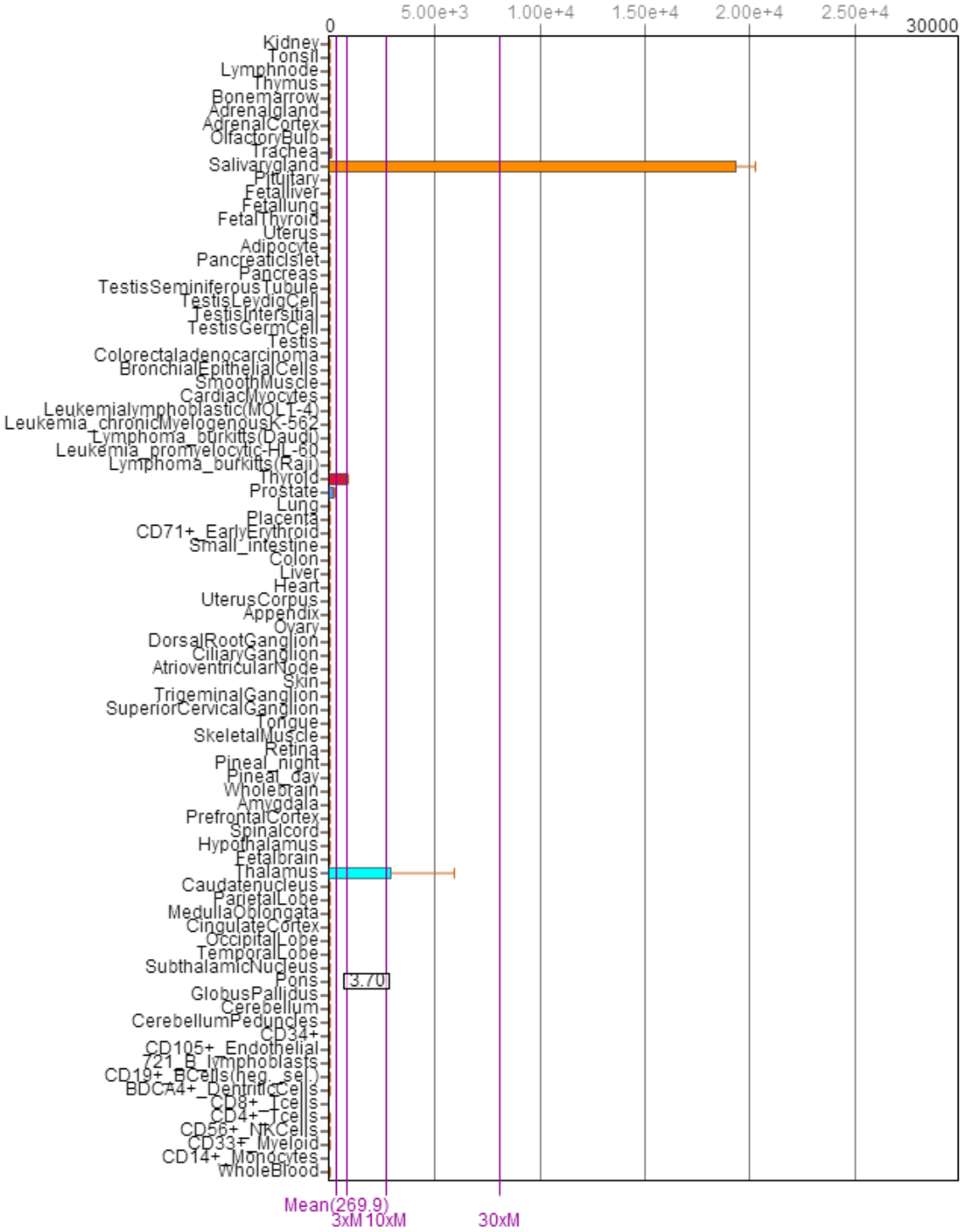


Appendix 5

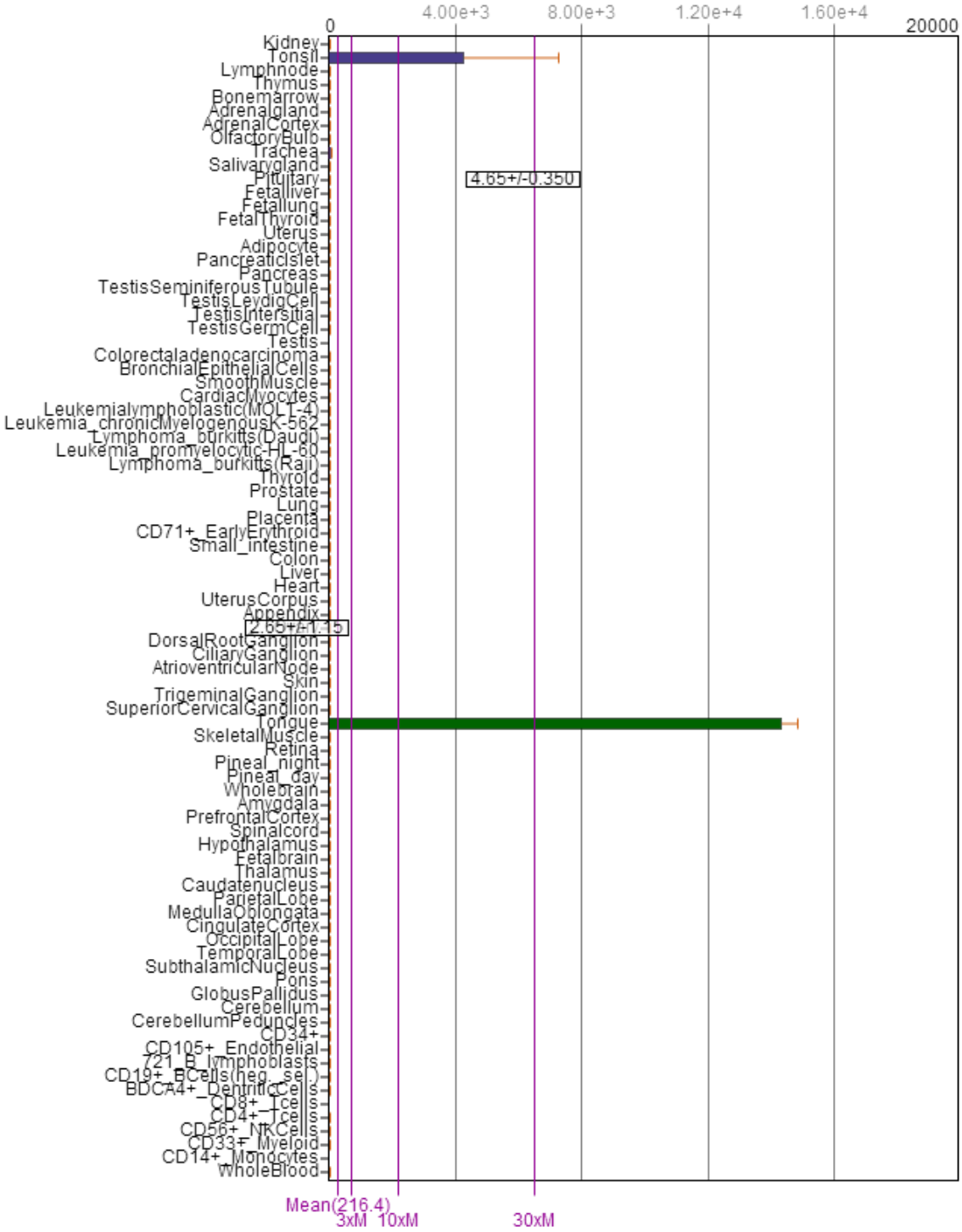
The expression of saliva-specific markers in different body fluids and tissues shown in BioGPS database.



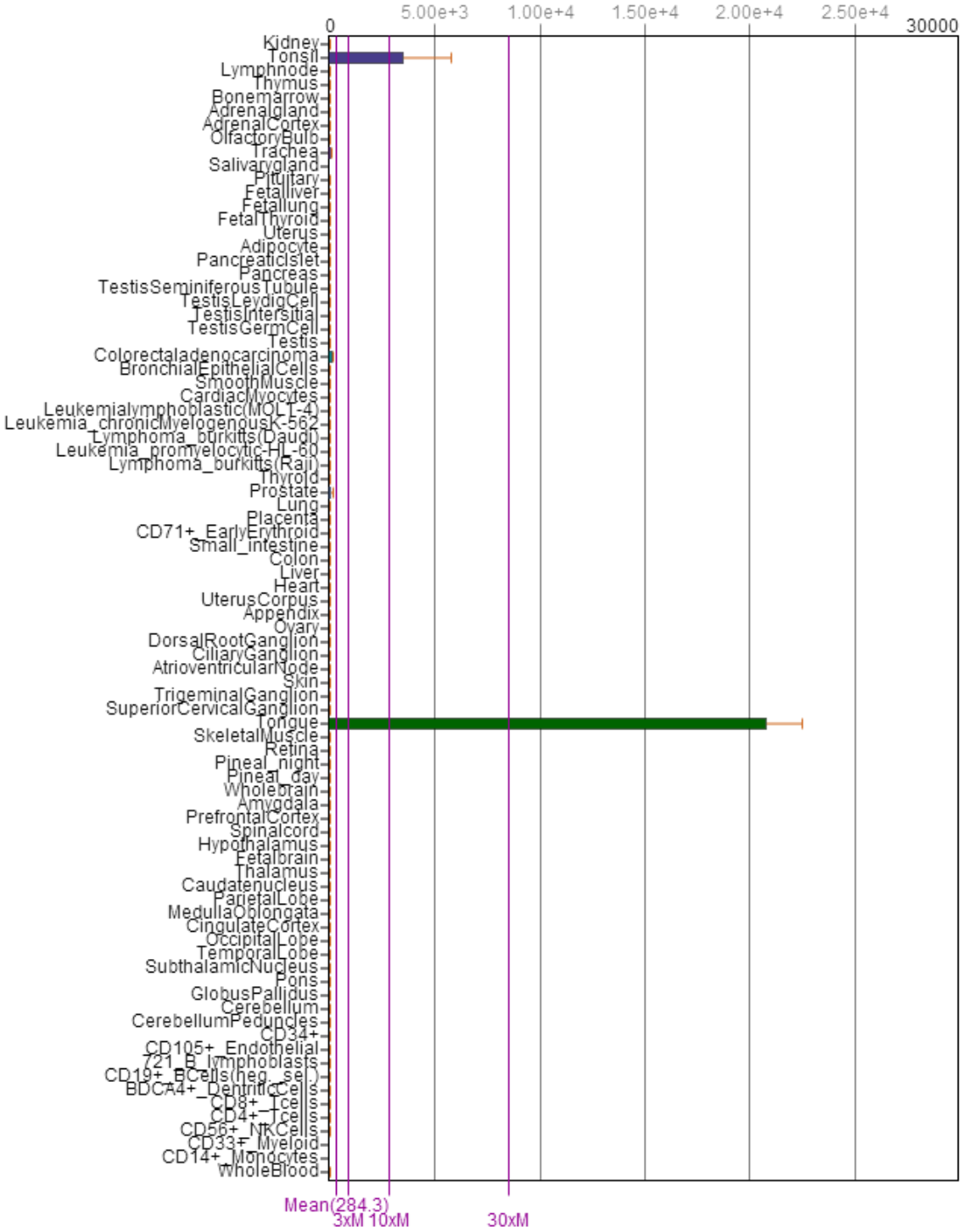
The expression of *STATH* in different body fluids and tissues shown in BioGPS database



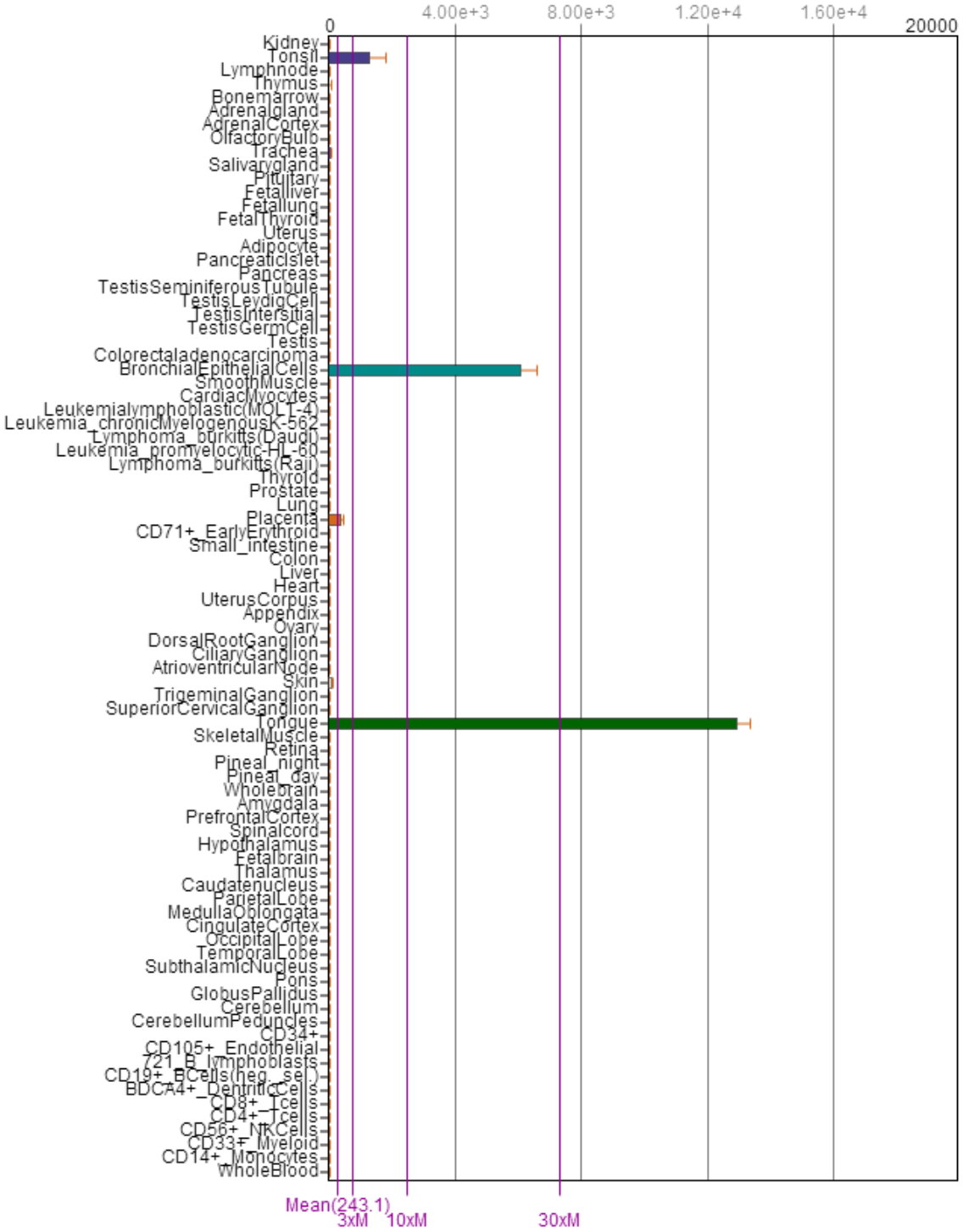
The expression of *HTN3* in different body fluids and tissues shown in BioGPS database



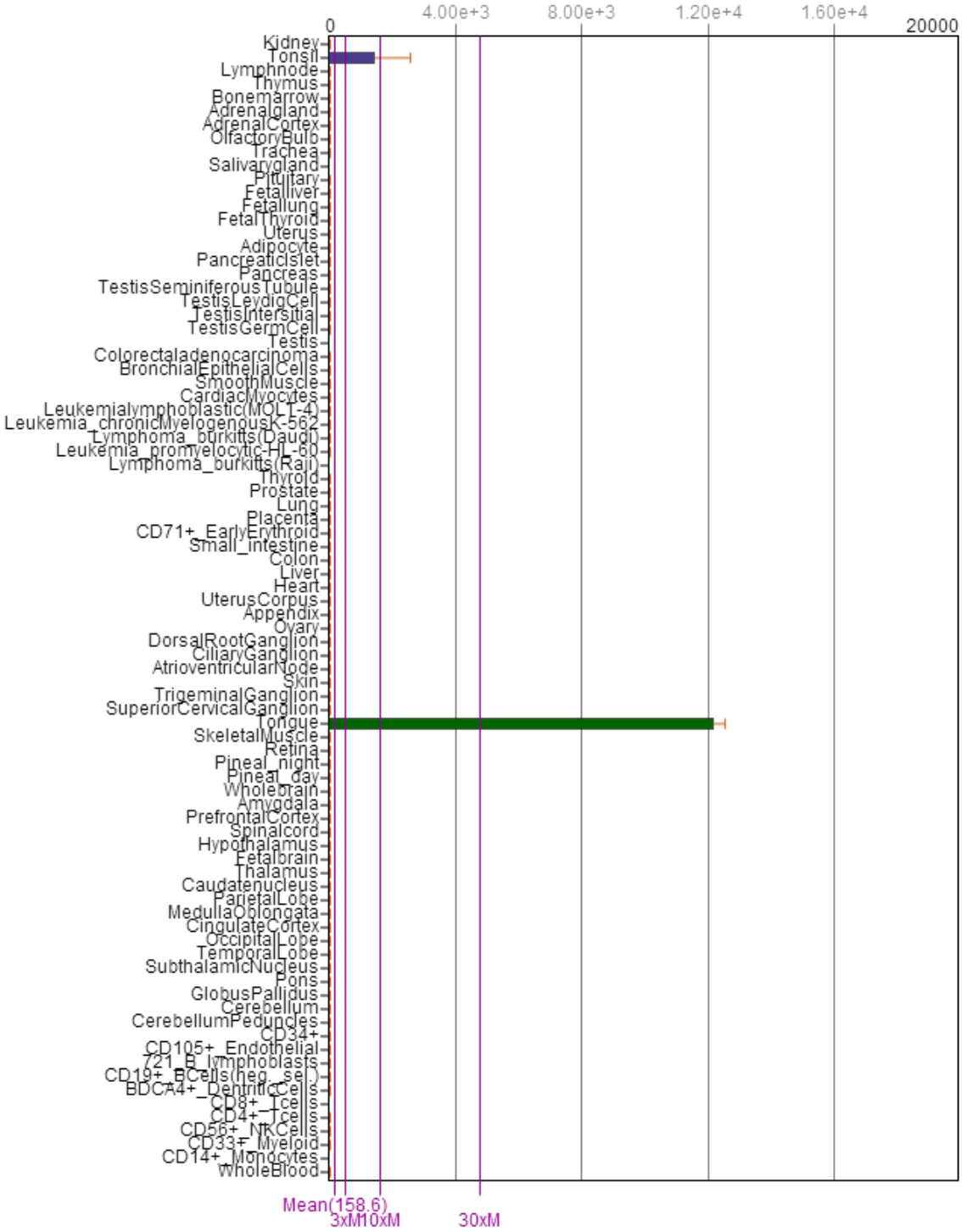
The expression of *KRT4* in different body fluids and tissues shown in BioGPS database



The expression of *KRT13* in different body fluids and tissues shown in BioGPS database



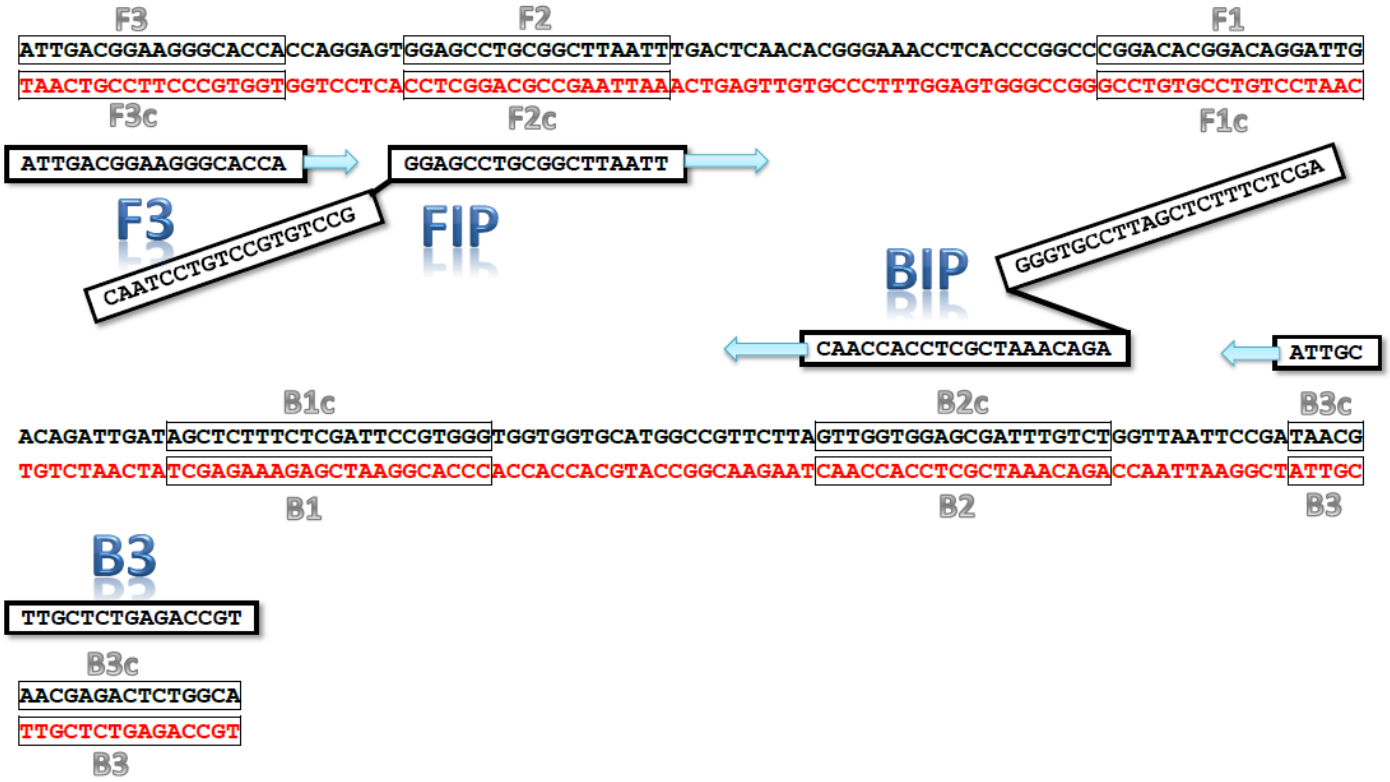
The expression of *KRT6A* in different body fluids and tissues shown in BioGPS database



The expression of *SPRR1A* in different body fluids and tissues shown in BioGPS database

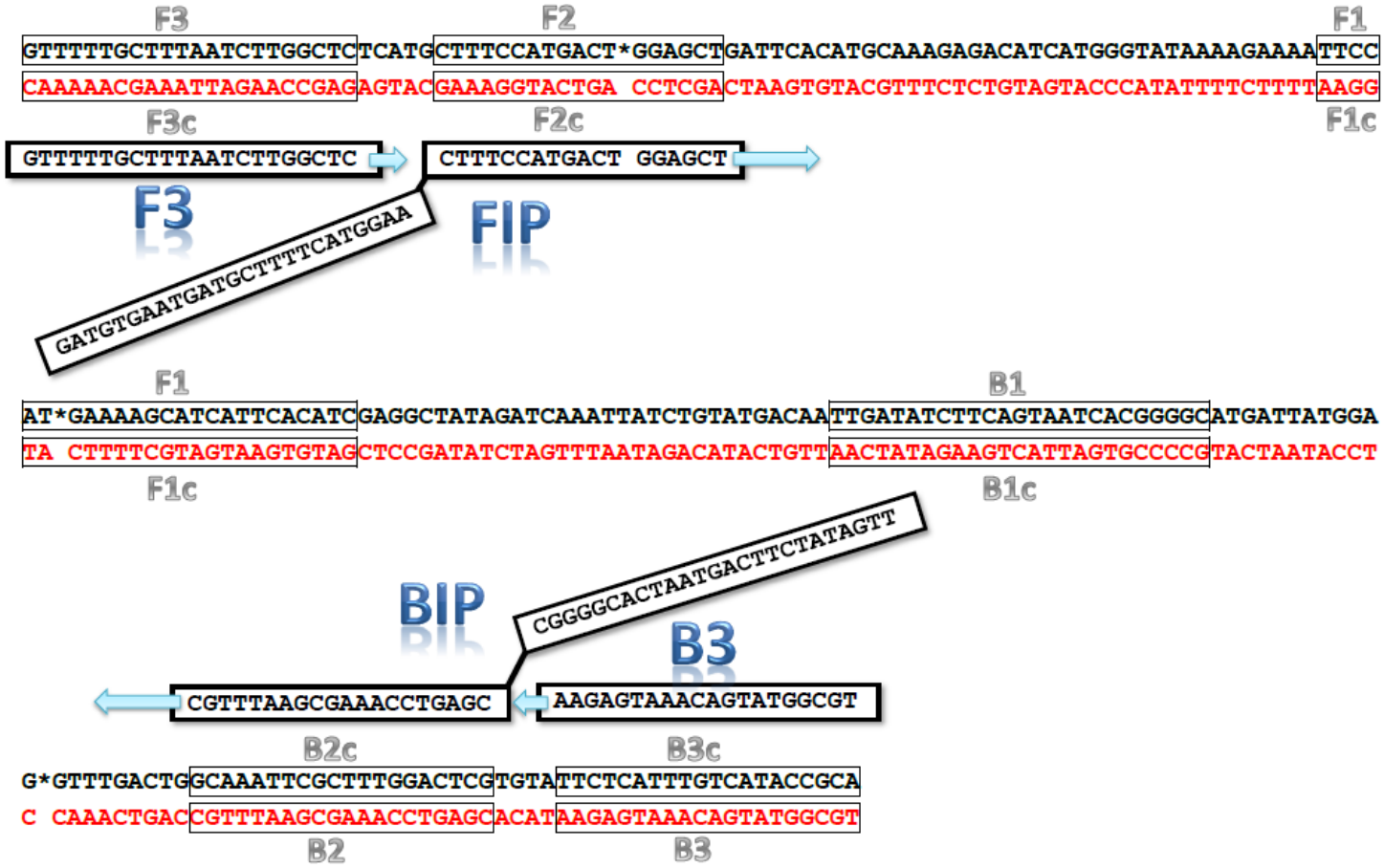
Appendix 6

Human 18S ribosomal RNA　　Target region：1199-1395(196mer)



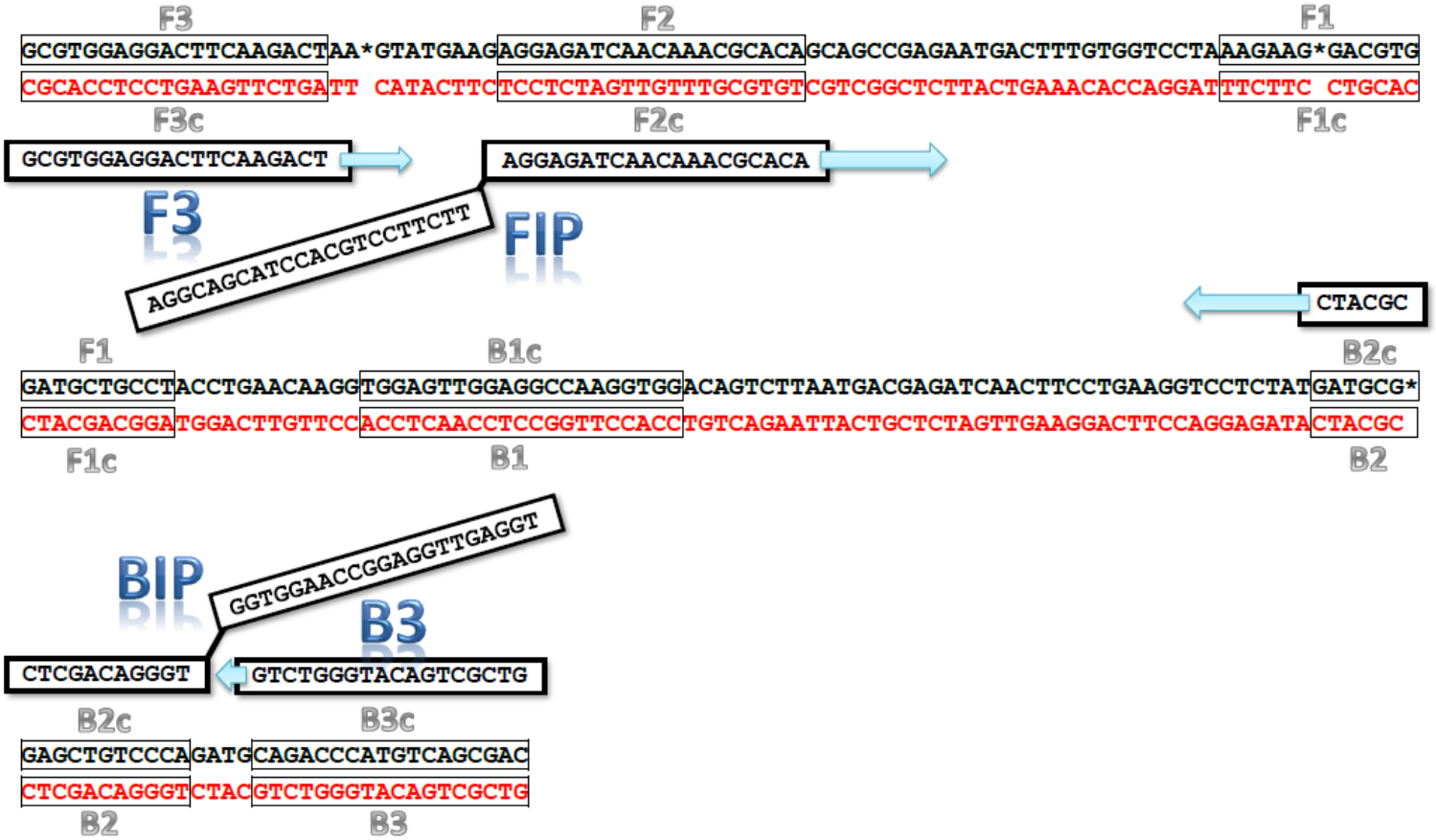
The sequences and target sites of *18S rRNA* LAMP primers

Homo sapiens histatin 3 (*HTN3*), mRNA　　Target region：120-349 (229mer)



The sequences and target sites of *HTN3* LAMP primers

Homo sapiens keratin 4 (*KRT4*), mRNA　　Target region：714-925 (212mer)



The sequences and target sites of *KRT4* LAMP primers

Appendix 7

Threshold time (Tt) of different body fluids using *18S rRNA* marker

Sample ID*	Tt (min)						
	Blood (n=13)	Saliva (n=14)	Semen (n=17)	Menstrual blood (n=12)	Sweat (n=14)	Urine (n=12)	Vaginal secretion (n=15)
1	33.8	38.4	26.8	36.7	41.9	52.6	50.9
2	42.1	38.8	37.7	31.0	47.9	44.1	47.5
3	46.8	43.2	39.4	33.9	57.3	52.6	45.4
4	45.4	35.3	47.2	44.3	57.4	53.4	53.6
5	37.7	32.3	42.8	35.8	40.0	45.3	36.7
6	49.9	39.0	26.3	35.4	39.7	42.0	43.5
7	42.5	30.7	42.3	39.9	55.1	55.9	39.2
8	30.4	45.2	41.6	39.8	34.1	46.5	41.1
9	52.0	35.7	30.1	43.9	37.3	44.8	46.0
10	32.9	37.6	29.4	32.5	54.2	46.3	49.7
11	34.8	35.1	29.1	33.9	34.4	40.9	44.0
12	45.9	36.5	30.2	30.7	55.6	42.8	43.0
13	31.3	35.8	59.3		41.6		37.1
14		48.2	29.9		39.5		44.4
15			41.0				51.1
16			51.5				
17			35.6				
Avg±S.D.	40.4±7.4	38.0±4.8	37.7±9.3	36.5±4.6	45.4±8.8	47.3±5.0	44.9±5.1

* The same sample ID did not mean the different body fluids were collected from the same individual.

Threshold time (Tt) of different body fluids using *HTN3* marker

Sample ID*	Tt(min)						
	Blood (n=13)	Saliva (n=14)	Semen (n=17)	Menstrual blood (n=12)	Sweat (n=14[#])	Urine (n=12)	Vaginal secretion (n=15)
1	-	46.8	-	-	-	-	-
2	-	39.9	-	-	-	-	-
3	-	34.5	-	-	-	-	-
4	-	40.0	-	-	-	-	-
5	-	45.1	-	-	-	-	-
6	-	42.4	-	-	-	-	-
7	-	38.0	-	-	-	-	-
8	-	37.8	-	-	-	-	-
9	-	36.9	-	-	-	-	-
10	-	37.8	-	-	-	-	-
11	-	41.0	-	-	-	-	-
12	-	45.1	-	-	-	-	-
13	-	38.1	-		-		-
14		43.3	-		-		-
15			-		40.0[#]		-
16			-				
17			-				
Avg±S.D.	-	40.5±3.6	-	-	-	-	

* The same sample ID did not mean the different body fluids were collected from the same individual.

This sweat sample was subsequently confirmed as a contamination with saliva when collecting the sweat and removed from the following analysis.

Threshold time (Tt) of different body fluids using *KRT4* marker

Sample ID*	Tt (min)						
	Blood (n=13)	Saliva (n=14)	Semen (n=17)	Menstrual blood (n=12)	Sweat (n=14)	Urine (n=12)	Vaginal secretion (n=15)
1	-	49.3	-	-	-	39.2	-
2	-	39.2	-	40.4	-	-	-
3	-	36.6	-	36.6	-	-	58.2
4	-	37.3	-	59.6	-	-	-
5	-	37.3	-	-	-	35.5	41.5
6	-	40.3	-	-	-	-	-
7	-	39.5	47.6	45.8	-	39.8	47.6
8	-	42.0	-	42.6	-	44.2	43.7
9	-	41.0	-	49.3	-	43.0	-
10	-	34.8	-	-	-	45.8	-
11	-	42.8	-	43.9	-	38.2	-
12	-	34.4	-	-	-	43.7	50.3
13	-	37.3	-		-		47.3
14		39.2	-		-		42.2
15			-				-
16			-				
17			-				
Avg±S.D.	-	39.4±3.8	-	-	-	-	-

* The same sample ID did not mean the different body fluids were collected from the same individual.

Appendix 8

The average of dCq for each time point for each donor

Time point (day)	Average of dCq							
	Donor1	Donor2	Donor3	Donor4	Donor5	Donor6	Donor7	Donor8
0	12.3	12.5	13.0	17.3	10.7	15.6	13.6	11.9
7	13.8	13.0	13.5	21.0	12.2	16.0	14.1	14.0
14	16.1	13.8	16.3	19.3	14.0	16.1	15.8	13.5
21	18.6	14.2	18.1	17.9	17.5	18.5	17.2	14.1
28	18.0	15.9	16.5	17.1	15.6	18.5	17.8	15.2
35	18.7	16.6	18.7	18.0	15.5	19.0	16.7	15.1
42	19.2	17.6	18.9	16.1	15.3	18.3	17.3	17.7
49	20.1	15.5	18.5	20.3	16.3	18.7	18.0	15.2
56	18.7	15.7	17.6	18.2	16.2	18.7	17.2	15.5